of HAV corresponding to amino acids 1520 to 1738; and conservative variations thereof, wherein the antigenically reactive HAV peptide is not identical to a HAV polyprotein and wherein the antigenically reactive peptide binds to an antibody specifically antigenically reactive with a peptide selected from the group consisting of SEQ ID NOS: 11-72 and conservative variations thereof.

82. (New) The antigenically reactive HAV peptide of Claim 81, wherein the conservative variations thereof consist of individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids.

REMARKS

Claims 70-72 and 77-82 are pending in this application after the withdrawal of claims 1, 69 and 74-76, due to the Applicants' election of Group II, the Examiner's withdrawal of claim 73 as being directed to a nonelected species, and pending entry of new claims 77-82. Attached hereto is a marked-up version of the changes made to the claims by the current amendment and a copy of the Abstract provided on a separate sheet as required in the Office Action. The attached page showing changes to the claims is captioned "Version with markings to show changes made." No new matter is believed added. Support for these amendments and the new claims can be found throughout the specification, as set forth below. In light of the following remarks, Applicants respectfully request reconsideration of this application and allowance of the pending claims to issue.

Applicants respectfully acknowledge the Examiner's consideration of the amendment filed

August 20, 2001, but have concerns whether the Applicants' arguments could have been fully considered. For, while the Office Action states that Applicant's arguments have been fully considered, the Examiner states that "Applicant's statement regarding attached Exhibits A-D with the Khudyakov et al. reference is noted; however no such exhibits were found with the response." These Exhibits were provided for their explicit teaching of the state of the art at the time the invention was made, which was that the nonstructural proteins were not immunogenic. Applicants respectfully assert that their argument, that one of skill in the art would not have been motivated to make the present invention, could not possibly been given full and proper consideration if these Exhibits were misplaced and so not considered. Applicants have again included these Exhibits with the present response along with a copy of the originally filed postcard indicating their transmission to the U.S. Patent and Trademark Office. However, in light of the failure of the Office to consider these references, and the resulting failure to fully consider Applicants' arguments, withdrawal of the finality of the present Office Action is believed merited and is respectfully requested.

I. Rejection under 35 U.S.C. § 112, second paragraph

Claims 70-72 remain rejected under 35 U.S.C. § 112, second paragraph, as allegedly not particularly pointing out and distinctly claiming the subject matter which the Applicants regard as their invention.

A. Specifically, the Office Action maintains that the rejection for "substantially similar to a portion," is not overcome by the Applicants' arguments as those arguments were not found to be

persuasive. It is stated that the specification at page 4, line 42, through page 5, line 26, pointed-out by the Applicants, "does not set forth the metes and bounds of the encompassed peptides other than to disclose that the peptides bind to an antibody which is immunoreactive with one of the peptides listed in the disclosure." Further, the Office Action states that "it does not set forth the *structural* metes and bounds of such peptides, but merely defines a single property of the peptides" (emphasis added). This, the Examiner asserts, fails to delineate what specific peptides are encompassed.

Applicants respectfully disagree that the disclosure to which the Examiner refers is inadequate to render the metes and bounds of the claims determined and definite. Furthermore, Applicants submit that the Examiner's assertion that the specification "does not set forth the *structural* metes and bounds of such peptides, but merely defines a single property of the peptides" (emphasis added) states an inappropriate standard. As outlined in the MPEP 2173.05(g), the use of functional limitations does not, by itself, render claims indefinite. Indeed, the line drawn between what is claimed, "peptides which bind," and what is not claimed, peptides which don't bind, is no less distinct than the analogous line drawn in *In re Barr*, wherein the line was drawn between those compounds that do not react and those that do. As stated in the MPEP, " the limitation used to define a radical on a chemical compound as "incapable of forming a dye with said oxidizing developing agent" although functional, was perfectly acceptable because it set definite boundaries on the patent protection sought. *In re Barr*, 444 F.2d 588, 1709 USPQ 33 (CCPA 1971)." As such functional limitations are clearly acceptable, further limitation to particular structures, as it appears the Examiner is suggesting, is not necessary. Applicants request removal of this basis of rejection.

Amended claims 70-72 and new claims 77-82 do not recite "substantially similar to a portion" and are not, therefore, subject to the rejection on the above-indicated grounds. Applicants, therefore, request both their entry and their allowance to issue.

B. Specifically, the Office action maintains that use of the term "portion" renders the claims indefinite. While the Applicants' prior argument that a "portion" indicates any fraction up to and including the complete item, such as a portion of the P2A protein, is acknowledged, the Examiner maintains that because a "portion" could encompass any fraction of a protein, it remains unclear what the claimed portions, fragments or fractions encompass.

Applicants respectfully disagree that the use of the word "portion" renders the claims indefinite. As the claims specifically recite a functional limitation, namely, that the peptide binds specifically to a defined antibody, these claims are definite. Applicants would again refer the Examiner to *In re Barr* and note that, as before, further limitation to particular structures, as it appears the Examiner is suggesting, is not necessary. As written, the present claims limited to peptides with a defined functional limitation are no less definite than the claimed invention at issue in *In re Barr*.

However, while Applicants maintain that no such structural guidance is required to provide adequate definiteness for the claimed invention, the present application does provide significant description of the structure of the claimed peptides. Specifically, the claimed peptides are all

defined by the amino acid sequence of the HAV polyprotein explicitly disclosed or conservative variations thereof, albeit in many overlapping portions/sequences, in the application as filed (e.g., SEQ ID NOS:1-72).

Furthermore, the present application provides 72 specific examples (SEQ ID NOS:1-72) of HAV peptides that are substantially similar to a "portion" of the disclosed sequence. Applicants submit that this is an adequate amount of guidance, with the additional teaching of the application, for those of skill in the art to recognize what is intended by the claimed invention. Applicants request removal of this basis of rejection from all rejected claims. Further, Applicants request that this basis of rejection not be applied to new claims 77-82, but that new claims 77-82 be entered and allowed to issue.

C. Specifically, the Office Action maintains that use of the phrase "conservative variations" renders the claims indefinite.

In regard to the Office Actions' assertion that the term "conservative variations thereof" is not defined in the disclosure, the term "conservatively modified variations" (page 13, lines 14-19) is used in the specification to describe nucleic acids which encode amino acid sequences with low levels of changes in the encoded amino acid sequence. Furthermore, the application includes "conservative substitution tables" which provide examples of conservative amino acid substitutions (page 13, lines 19-28) and further states that the amino acids can include "individual subsitutions,

deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids" (page 13, lines 14-28). Together with the knowledge in the art, these teachings in the specification render the intended meaning of conservative variations of amino acid sequence quite definite to those of skill in the art. Thus, the term "conservative variations," when used in context of encoded amino acid sequence, clearly conveys to those of skill in the art that the amino acid sequences were those encoded by, or which could be encoded by, "conservatively modified variations" of nucleic acid sequences as defined in the specification. Correspondingly, none of the claims reciting "conservative variations thereof," specifically claims 70, 77, 79 and 81, nor any claims dependent therefrom, are indefinite.

Further, Applicants submit that claim 71 as amended and new claims 80 and 82 reciting "...wherein the amino acid sequence can include individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids" (page 13, lines 14-28) is not indefinite. Support for the amendment and for these new claims, in particular the limitation to what variation can be allowed in the amino acid sequence, can clearly be found in the specification as filed.

Further, claim 72 as amended is further limited so as to no longer recites "conservative variations thereof." Correspondingly, Applicants submit that the rejection on that basis is moot.

Applicants request removal of this rejection from claims 70-72. Further, Applicants submit

that it is inappropriate, for the reasons indicated above, to reject new claims 77-82 on these same grounds. Applicants therefore request both the entry of new claims 77-82 and their allowance to issue.

D. Specifically, the Office Action maintains that the applicant did not point out any reference sequence upon which the numbering system was based. Further, the Examiner maintains that the disclosure referenced in Tables 2-11 fails to provide such a reference sequence and that absent such a reference sequence, the metes and bounds of the claimed invention cannot be ascertained and the claims are indefinite.

Applicants submit that the present application, as filed, indicates that the peptides of the invention are derived from a single polyprotein that is subsequently processed into structural and nonstructural proteins (page 1, lines 18-23). The order that these structural and nonstructural proteins are arranged in the polyprotein is clearly defined by the amino acid numbering given to these proteins and corresponds to the known arrangement of the portions that are processed from the nascent polyprotein. Specifically, from the amino terminus to the carboxy terminus, the polyprotein includes the VP4, VP2, VP3, VP1, P2A, P2B, P2C, P3A, P3B, P3C and P3D proteins. As is indicated in the titles of Tables 2-11, each of these proteins corresponds to a certain region, defined in terms of amino acid residues, e.g., in the title for Table 2, peptides are "derived from the VP4 (1-23 aa) and VP2 (24-245 aa)." The sequential arrangement of the proteins in the tables, the corresponding sequential notation of the amino acids contained within those proteins (denoted by

the commonly accepted usage of "aa" for amino acid residue), and consistent usage of the numbering derived from the full-length polyprotein convey that the numbering is based on the full-length polyprotein. This usage of the originally expressed polyprotein's amino acid sequence as the basis for the numbering of any portion of sequence derived from the full-length polyprotein is clear to one of skill in the art given the present disclosure as filed. Furthermore, as HAV appears to consist of a single serotype and lacks any significant antigenic variation (page 1, lines 22-23), one of skill in the art can identify any analogous portions of the polyprotein, or processed portions thereof, and any peptides derived therefrom from the nomenclature used herein. Indeed, this same system of nomenclature is used in the Chiron reference cited against the present application for allegedly rendering the present claims obvious (European Patent Application 0 199 480; see for example, column 34, lines 18-22, and Figure 1). The numbering system used in the present application, the same as that used in the cited reference, does not render any claims of the present application indefinite. Applicants, therefore, respectfully request removal of this basis of rejection from pending claims 70-72 and that this basis of rejection not be applied to new claims 77-82.

II. Rejection under 35 U.S.C. § 103

The rejection of claims 70-72 under 35 U.S.C. § 103(a) as unpatentable over Chiron is maintained. The Office Action states that the Applicant's arguments that Chiron neither made nor tested any peptide corresponding to AA 792-848 of HAV were considered, but were not found persuasive. Specifically, it is stated that the Applicants' claims do not require that the peptide correspond to AA 792-848, but rather only requires that the peptide be substantially similar. It is

also stated that the Applicants' arguments that the nonstructural proteins are not immunogenic are not relevant to the claims as the claims are drawn to antigenically reactive peptides, not to immunogenic peptides. Further, the Examiner notes that the Applicants' argument that Khudyakov et al. establishes that the P2A nonstructural proteins were not taught in the art to be immunogenic is not understood, as the applicant has not pointed out where in the reference such a teaching is found. Rather, the Examiner points to the last sentence of the abstract in the Khudyakov et al. reference wherein it states "collectively these data demonstrate that HAV structural and nonstructural proteins contain antigenic epitopes that can be efficiently modeled with short synthetic peptides." This statement, the Examiner alleges "would teach toward a reasonable expectation of success in producing the peptides of the claimed invention."

Applicants submit that none of the claims, including new claims 77-82 are obvious over Chiron and, correspondingly, all of claims 70-72 and 77-82 are patentable. As noted in the prior Response filed on August 20, 2001, while Chiron discloses the entire genome of HAV and recites a preferred immunogenic peptide as derived from AA 792-848, Chiron does not demonstrate the existence of any isolated immunogenic peptide derived from AA 792-848. As is taught in the prophetic example in Chiron, a nucleic acid sequence encoding amino acids 792-848 is "synthesized....[and] cloned into ..[a] vector.. [thereby] replacing a 150 bp fragment of the HbsAg coding for a dominant epitope." (Column 31, lines 44-51). However, Chiron neither made nor tested any peptide corresponding to AA 792-848. Yet, it is over that peptide that it is alleged the present invention is both obvious and unpatentable. The basis for this allegation is that Chiron

provides the complete sequence of HAV and teaches a preferred immunogenic peptide from the region AA 792-848. However, as was noted in the previous Response, Chiron was wrong and, prior to the filing of the present application, the skilled person recognized that Chiron was wrong. The region AA 792-848, corresponding to non-structural protein P2A, was not immunogenic. However, because Chiron did not actually make any peptide from this region, this fact was not recognized at the time. Subsequent to Chiron, but prior to the conception of the present invention, the non-immunogenic character of the P2A protein, and of the other non-structural proteins, was recognized by those of skill in the art.

The references provided as Exhibits A-D provide evidence of that recognition of the non-immunogenic nature of the non-structural proteins. While Exhibit A (Khudyakov et al.) does include statements that the Examiner asserts are suggestive of the present invention, Khudyakov et al. was published after the present application was filed and, as such, does not represent the knowledge of those of skill in the art at the time the invention was made. Rather, Khudyakov et al. presents the finding and use of peptides like those presently claimed and refers to other teaching in the art which does pre-date the present invention (including; Robertson et al., *Vaccine* 10: S106-S109 (1992); Robertson et al., *J. Med. Virology* 40: 76-82 (1993); and Jia et al., *J. Infect. Diseases* 165: 273-280 (1992), provided as Exhibits B, C & D, respectively). It is these references, not Khudyakov et al. (Exhibit A), that illustrates the knowledge and understanding of one of skill in the art over which the obviousness of the present invention should be determined. Applicants submit that any such determination will not find the present invention obvious, as Chiron was clearly

shown in the art to be incorrect in teaching that non-structural proteins are immunogenic,and thus could not provide a reasonable expectation of success for one of skill in the art. Thus, the stated rejection does not fulfil the minimal requirements of a *prima facie* case of obviousness. Applicants therefore request removal of this basis of rejection.

Furthermore, Applicants submit that Chiron does not suggest any HAV peptide containing less than the complete portion corresponding to amino acids 792-848. The only recitation of amino acids 792-848 is in the context of inclusion of this entire sequence as an insertion/substitution in the hepatitis B surface antigen (column 31, lines 26-29). As inclusion of a different portion of differing size or sequence would be expected to result in different properties, the cited recitation of this sequence can do no more than render obvious an identical composition containing the same sequence inserted into a hepatitis B surface antigen. This is particularly true as one of skill in the art would be aware that properties conferred to the disclosed fusion by the hepatitis B derived sequence would be expected to greatly influence the antigen-like character of the fusion. Indeed, this expectation is discussed in Chiron itself at column 10, lines 10-14, wherein it states "[t]he immunogenicity of the epitopes of HAV may also be enhanced by preparing them in mammalian or yeast systems fused with particle-forming proteins such as that associated with hepatitis B surface antigen." Correspondingly, given the altered properties that the extraneous hepatitis B derived sequence would most likely, and was fully expected to, confer, Applicants submit that any suggestion or teaching that this recitation may conceivably provide to one of skill in the art can only be in the context of inclusion of a sequence from HAV in a fusion protein with hepatitis B surface

antigen. Further, Applicants submit that given the inclusion of a HAV sequence similar in size to that excised from the antigen, the teaching, if it can be afforded any weight, can also only be suggestive for the sequences very similar both in size and in sequence to that of amino acids 792-848.

Correspondingly, Applicants submit that pending claims 70-82 are not rendered obvious by the recitation of that single particular peptide sequence cited in Chiron, particularly given the teaching of the art regarding the lack of immunogenicity of nonstructural peptides, nor by that recitation in combination with a complete HAV polyprotein sequence. Further, claim 72 as amended, which recites specific sequences neither disclosed nor suggested in either Chiron or in the complete HAV polyprotein sequence, cannot be rendered obvious for the reasons outlined in *In re Bell*. Applicants therefore request removal of these grounds of rejection and allowance of claims 70-72 to issue.

It is well-recognized in the patent law that a claimed nucleic acid or amino acid is not obvious unless the cited art provides the motivation and specific guidance to obtain the claim molecule (*In re Bell* (26 U.S.P.Q. 2d, 1529-1532 (Fed. Cir.1993)). Thus, even if one were to accept the proposition that Chiron provides a general motivation to make a peptide fragment of the HAV polyprotein, it is absolutely clear that Chiron does not provide any guidance that points to any peptide other than the 792-848 fragment disclosed.

As is discussed in *In re Bell* (26 U.S.P.Q. 2d, 1529-1532 (Fed. Cir.1993), the sequence of a specific protein coding nucleic acid was not obvious despite the disclosure in the prior art of the amino acid sequence encoded by the claimed nucleic acid, and the unquestioned teaching in the art of how to identify each and every nucleic acid that could encode the protein. The rationale for this decision was that a claimed molecule is not obvious unless the art points to that molecule. It is not enough that the art would allow the skilled person to find the claimed molecule. This, Applicants submit, is analogous to the present case in respect to Chiron. The cited reference discloses a complete sequence of an HAV isolate, from which any number of particular peptides could be derived. Of the nearly infinite number of these particular peptides, the Office Action cites a polypeptide comprising a portion that corresponds to amino acids 792-848 of the HAV polyprotein.

Furthermore, no peptide claimed in claims 77-82 includes the sequence of the complete HAV polyprotein. The peptide of claims 77-79 can not include all portions of the complete polyprotein. The peptide of claims 80-82 must have an amino acid sequence different from that of the HAV polyprotein.

Further, no peptide claimed by any new claim corresponds to the portion of the P2A protein disclosed in Chiron. Specifically, the peptide of claim 77 must not contain the complete sequence of the portion of the P2A protein disclosed in Chiron. The peptide of claim 78 must not contain any portion of the portion of the sequence of the P2A protein disclosed in Chiron. The peptide of claim 79 must include additional sequence from at least one other HAV protein not disclosed in the

portion of the P2A protein disclosed in Chiron. The peptide of claim 80, dependent from claim 79, must also include sequence not contained in any HAV polyprotein. The peptides of claims 81 and 82 must include both at least a portion of the P2A protein, including that supposedly disclosed in Chiron, and portions of further proteins of the HAV polyproteins, wherein sequence included in the peptides of claims 81 and 82 are not contained in any HAV polyprotein.

As there is no teaching or suggestion in Chiron to make the necessary modifications to the sequences disclosed in Chiron, or the sequence of the full-length HAV polyprotein, Chiron fails to render the invention as claimed obvious. Most particularly, as the invention is claimed in claims 77-82. As discussed in the MPEP § 2143, two of the three basic requirements of a *prima facie* case of obviousness are that there must be suggestion or motivation in the references themselves, or in the knowledge available to one of ordinary skill in the art to modify the reference, and that the prior art teaching must teach or suggest all the claim limitations. Neither of these requirements for establishing a *prima facie* case of obviousness are provided by Chiron, either alone, or in combination with the complete sequence of the HAV polyprotein.

Therefore, Applicants submit that the newly added claims are unobvious and request, accordingly, both their entry and allowance to issue.

Pursuant to the above amendments and remarks, consideration and allowance of the pending application is believed warranted. The Examiner is invited and encouraged to directly contact the

undersigned if such contact may enhance the efficient prosecution of this application to issue.

Credit Card Form PTO-2038 is enclosed to cover the fee of $360.00 for the newly added claims submitted herewith. This amount is believed to be correct; however, the Commissioner is hereby authorized to charge any additional fees which may be required to Deposit Account No. 14-062.

Respectfully submitted,

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231, on the date shown below.

Gwendolyn D. Spratt — Date: 2-20-02

Version with Markings to Show Changes Made

In the claims:

70. (Twice amended) An isolated, antigenically reactive hepatitis A virus (HAV) peptide, the antigenically reactive peptide comprising an amino acid sequence [which is substantially similar to] of a portion of an HAV protein selected from the group consisting of the VP3 protein of HAV corresponding to amino acids 246 to 491; the VP1 protein of HAV corresponding to amino acids 492 to 791; the P2A protein of HAV corresponding to amino acids 792 to 980; the P2B protein of HAV corresponding to amino acids 981 to 1087; the P2C protein of HAV corresponding to amino acids 1088 to 1422; the P3A protein of HAV corresponding to amino acids 1423 to 1496; the P3B protein of HAV corresponding to amino acids 1497 to 1519; the P3C protein of HAV corresponding to amino acids 1520 to 1738; and conservative variations thereof, wherein the antigenically reactive peptide binds to an antibody specifically antigenically reactive with a peptide selected from the group consisting of SEQ ID NOS: 11-72 and conservative variations thereof.

71. (Twice amended) The antigenically reactive peptide of claim 70, wherein the amino acid sequence can include individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids, and wherein the antigenically reactive peptide binds to an antibody specifically antigenically reactive with a peptide selected from the group consisting of SEQ ID NOS: 11-72 [and conservative variations thereof].

72. (Twice amended) The antigenically reactive peptide of Claim 70 wherein the antigenically reactive peptide has an amino acid sequence selected from the group consisting of SEQ ID NOS: 11-72 [and conservative variations thereof] and wherein amino acid sequence from at least one of SEQ ID NOS:38-43 is excluded.

Abstract

Antigenically reactive regions of the Hepatitis A virus polyprotein are provided. These antigenically reactive regions, and polypeptides and proteins comprising these antigenically reactive regions, provide a sensitive and specific immunological hepatitis A virus detection assay. The specific use of regions derived from the nonstructural regions of the polypeptide provides the basis for determining immunity derived from prior or present infection by the Hepatitis A virus and allows one to distinguish between an immunological response derived from infection and that derived from immunization.

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EXHIBIT "A"

Virology 260, 260–272 (1999)
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Antigenic Epitopes of the Hepatitis A Virus Polyprotein

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Forty-two antigenic domains were identified across the hepatitis A virus (HAV) polyprotein by using a set of 237 overlapping 20-mer synthetic peptides spanning the entire HAV polyprotein and a panel of serum samples from acutely HAV-infected patients. The term "antigenic domain" is used in this study to define a protein region spanned with consecutive overlapping immunoreactive peptides. Nineteen antigenic domains were found within the structural proteins, and 22 were found within the nonstructural proteins, with 1 domain spanning the junction of VP1 and P2A proteins. Five of these domains were considered immunodominant, as judged by both the breadth and the strength of their immunoreactivity. One domain is located within the VP2 protein at position 57–80 aa. A second domain, located at position 767–842 aa, contains the C-terminal part of the VP1 protein and the entire P2A protein. A third domain, located at position 1403–1456 aa, comprises the C-terminal part of the P2C protein and the N-terminal half of the P3A protein. The fourth domain, located at position 1500–1519 aa, includes almost the entire P3B, and the last domain, located at position 1719–1764 aa, contains the C-terminal region of the P3C protein and the N-terminal region of the P3D protein. It is interesting to note that four of the five most immunoreactive domains are derived from small HAV proteins and/or encompass protein cleavage sites separating different HAV proteins. The HAV-specific immunoreactivity of each antigenically reactive peptide was confirmed by using seven HAV seroconversion panels. Collectively, these data demonstrate that HAV structural and nonstructural proteins contain antigenic epitopes that can be efficiently modeled with short synthetic peptides.

INTRODUCTION

Hepatitis A virus (HAV), the causative agent of hepatitis A, is a unique member of the family *Picornaviridae* (Gust *et al.*, 1983; Minor, 1991). The HAV genome is a positive single-stranded RNA, 7.5 kb in length. Similar to all *Picornaviridae*, the HAV genome contains one large open reading frame encoding a polyprotein, which is eventually cleaved into smaller structural and nonstructural proteins (Hollinger and Ticehurst, 1996).

The antigenic composition of the intact virions has been thoroughly studied. Multiple antigenic epitopes have been detected on the surface of virions and 14S pentamer subunits by using monoclonal antibodies. It was shown that almost all epitopes are located within one immunodominant neutralization site (Lemon and Robertson, 1993; Ping and Lemon, 1992; Stapleton and Lemon, 1987); however, other studies using chimeric picornaviruses have suggested the existence of a secondary neutralization site near the N-terminus of VP1 (Lemon *et al.*, 1992). This finding confirmed a previous observation that peptide derived from the N-terminal regions of VP1 induced HAV neutralizing antibodies (Emini *et al.*, 1985). Most murine HAV monoclonal antibodies compete with one another, and these antibodies can substantially block polyclonal human antibodies from binding HAV in competition immunoassays (Hughes *et al.*, 1984; Stapleton and Lemon, 1987). These observations suggest that there are only a limited number of antigenic epitopes closely placed on the surface of virions. Polyclonal or monoclonal antibodies obtained against native HAV demonstrated only marginal reactivity with denatured capsid proteins (Lemon, 1992). Similarly, antibodies raised to purified capsid proteins VP1, VP2, and VP3 did not efficiently neutralize HAV (Hughes and Stanton, 1985). These observations led to the suggestion that the antigenic epitopes of HAV are mainly conformational discontinuous structures that assemble by higher order interactions between capsid proteins (Lemon and Ping, 1988; Lemon, 1992).

Several approaches have been taken to map the HAV neutralizing epitopes. By cross-linking the Fab fragment of one HAV neutralizing monoclonal antibody to the VP1 protein in intact virions, it was shown that this protein plays an essential role in forming the HAV neutralizing epitope (Hughes *et al.*, 1984). Another approach was to

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0042-6822/99

identify mutations within the HAV capsid proteins that result in resistance to neutralization with monoclonal antibodies. Neutralization escape mutations for the HAV strain HM175 were identified at Asp_{70}, Gln_{74} of the VP3 protein, Ser_{102}, Val_{171}, Ala_{175}, and Lys_{221} of the VP1 protein (Ping *et al.*, 1988; Ping and Lemon, 1992), and for strain HAS15 at Pro_{65}, Asp_{70}, and Ser_{71} of VP3 and at Asn_{104}, Lys_{105}, and Gln_{233} of the VP1 protein (Nainan *et al.*, 1992).

Synthetic peptides have been extensively used to identify diagnostically relevant antigenic epitopes within the structural proteins (Bosch *et al.*, 1998; Chikin *et al.*, 1991; Kulik *et al.*, 1994, 1995); however, all attempts to find these epitopes have been unsuccessful (Bosch *et al.*, 1998; Chikin *et al.*, 1991; Kulik *et al.*, 1994, 1995). This observation was considered as additional evidence of the strict conformational nature of the HAV capsid antigenic sites. In order to overcome this conformational limitation on modeling HAV epitopes with synthetic peptides a new approach based on selection of desired immunoreactive peptides from random sequence peptide libraries (Houghten *et al.*, 1991; Scott and Smith, 1990) was employed to discover a mimotope that reacted with monoclonal and polyclonal anti-HAV antibodies (Mattioli *et al.*, 1995). A synthetic peptide containing this mimotope very efficiently detected IgM anti-HAV activity in serum specimens of acutely infected patients, although it failed to efficiently detect IgG antibodies (Mattioli *et al.*, 1995).

The HAV nonstructural proteins are also antigenically reactive. An assay based on immune precipitation of proteins translated *in vitro* has been developed to detect antibodies against the HAV nonstructural proteins (Jia *et al.*, 1992). This assay was used to discriminate between antibodies after natural infection and vaccination (Robertson *et al.*, 1992, 1993). Antibodies against P2 proteins were found in all sera from acutely infected patients, whereas chimpanzees vaccinated with inactivated or cell-adapted HAV had no detectable antibodies against P2 products (Robertson *et al.*, 1993). In another study, an enzyme immunoassay (EIA) was developed to detect antibodies to the nonstructural P3C protein. Antibodies to this protein were detected in the serum of primates experimentally infected with virulent HAV and in the serum of acutely infected patients, but antibodies to P3C protein were not detected in the serum from primates following immunization with inactivated HAV (Stewart *et al.*, 1997). These findings demonstrate the utility of HAV nonstructural proteins in differentiating inactivated vaccine-induced immunity from natural infection. However, no further studies on the antigenic composition of these proteins were conducted.

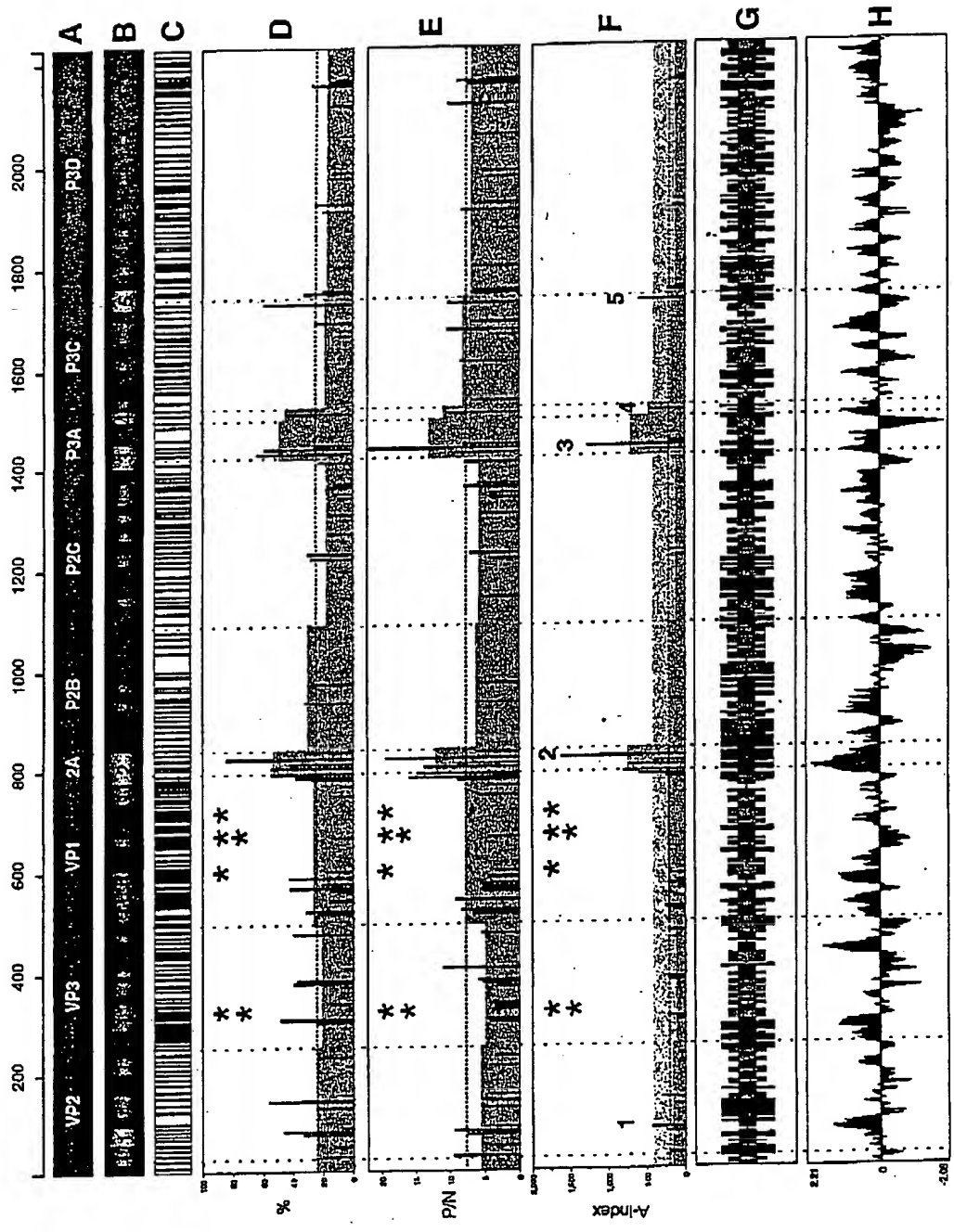
HAV infection is diagnosed by the detection of IgM or IgG antibodies to the capsid proteins (Bradley *et al.*, 1977). Because of apparently poor antigenic reactivity, HAV recombinant proteins and synthetic peptides have not been successfully employed in an EIA for the detection of anti-HAV activity. Currently, the only available source of immunoreactive proteins for assay development is inactivated HAV derived from cell culture, which is currently used by all commercial companies that manufacture anti-HAV tests. In addition to the inconvenience and cost associated with the production, purification, and standardization of cell culture-derived HAV antigen, current commercially available assays are unable to discriminate between natural infections and vaccine induced immunity, as emphasized in several publications (Jia *et al.*, 1992; Robertson *et al.*, 1992, 1993; Steward *et al.*, 1997), since these tests utilize intact HAV and therefore will detect antibodies in either case.

The present study provides, for the first time, a comprehensive analysis of the antigenic composition of the entire HAV polyprotein by using a large number of synthetic peptides. The results demonstrate the existence of several broadly and strongly antigenically reactive regions, many of which have significant diagnostic potential within both structural and nonstructural proteins.

RESULTS AND DISCUSSION

Peptide design

The sequence of the polyprotein of the HAV from strain HM175 (Cohen *et al.*, 1987) was used to design all synthetic peptides in this study. A total of 237 overlapping 20-mer peptides were synthesized. Almost the entire sequence of the HAV HM175 polyprotein was spanned with these peptides with only a few exceptions: four small hydrophobic regions at positions 106–109, 1011–1029, 1471–1476, and 1546–1547 aa were not spanned with peptides. In addition, in three cases peptides were derived from adjacent nonoverlapping hydrophobic regions in such a way that positions 980–981, 1087–1088, and 1107–1108 aa were represented only at the termini of peptides. These seven regions are shown in Fig. 1C as larger than average gaps between small vertical bars. Some regions where antigenic activity has been shown previously (Emini *et al.*, 1985; Kulik *et al.*, 1994, 1995; Nainan *et al.*, 1992; Ping *et al.*, 1988; Ping and Lemon, 1992) or could be predicted with high probability (Jameson and Wolf, 1988) based on protein chain flexibility (Karplus and Schultz, 1985), hydrophylicity (Kyte and Doolittle, 1982), and/or secondary structure (Chou, 1990) were spanned with a higher peptide density. These regions can be identified in Fig. 1C as clusters of small vertical bars. For example, two clusters of peptides within the N-terminal part of the VP3 protein (Fig. 1C) were generated around an antigenic epitope that had been previously reported (Kulik *et al.*, 1995) and around residues Asp_{315} and Gln_{318} (shown by two asterisks in Fig. 1C) that have been found to be essential to the HAV major conformational neutralizing epitope (Ping *et al.*, 1988; Ping and Lemon, 1992). Within the VP1 protein, peptide clusters were synthesized around an antigenic


A
B
C
D
E
F
G
H
VP2
VP3
VP1
2A
P2B
P2C
P3A
P3C
P3D
200
400
600
800
1000
1200
1400
1600
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2000
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P/N
A-index
1
2
3
4
5

epitope that had been previously found to elicit neutralizing antibody (Emini *et al.*, 1985; Lemon *et al.*, 1992); around amino acid residues Ser_{833}, Val_{662}, Ala_{657}, and Lys_{712} (indicated by asterisks in Fig. 1C), which were shown to be important for the functional activity of the major HAV neutralizing antigenic epitope (Ping and Lemon, 1992); and around the region at position 714–752 aa that was shown to be exposed on the surface of HAV particles (Robertson *et al.*, 1989). Surface exposure is generally considered to be an important indicator of potential antigenic reactivity (Emini *et al.*, 1985).

On average, every amino acid of the HAV HM175 polyprotein was represented 2.1 times in these synthesized peptides. Peptides were overlapped on average by 10 aa. In peptide clusters, peptides were often overlapped by 14–18 aa. Among 237 peptides, 110 were derived from structural proteins and 127 were derived from nonstructural proteins. Thus, taking into consideration the difference in the size of the HAV structural region (791 aa) and the nonstructural region (1436 aa), the density of peptides derived from structural proteins is approximately 1.6 times greater than that from nonstructural proteins.

Immunoreactivity of peptides derived from structural proteins

The antigenic reactivity of the HAV structural proteins has been extensively investigated using synthetic peptides (Bosch *et al.*, 1998; Chikin *et al.*, 1991; Kulik *et al.*, 1994, 1995), and strong antigenic regions modeled with short synthetic peptides or with recombinant proteins were not ascertained. In this study, however, a number of strong antigenic epitopes were discovered. These contradicting results may be explained by: (1) the use of acute-phase serum specimens in the present study, whereas all previous studies employed convalescent-phase serum specimens or polyclonal or monoclonal antibodies obtained against HAV particles and (2) a thorough scan of each protein by using significantly overlapping peptides.

VP4–VP2 proteins. Among 21 peptides derived from the VP4–VP2 proteins, 10 were found immunoreactive with acute anti-HAV serum specimens, as indicated by the vertical bars in Fig. 1. These immunoreactive peptides were used to identify 6 antigenic domains, as indicated by alternating consecutive rows of the same shade in Table 1. In this study, an antigenic domain is defined as a protein region spanned with immunoreactive consecutive overlapping synthetic peptides or a single peptide separated from other antigenic regions by nonimmunoreactive peptides. For example, four domains of the VP4–VP2 protein, at positions 15–34, 35–54, 194–213, and 214–233 aa, were identified with only one peptide each. Two domains at positions 57–90 and 110–156 aa were spanned with three peptides each (Table 1). Three peptides (1210, 1211, and 1216) from the two largest domains within the VP4–VP2 region demonstrated broad immunoreactivity with acute anti-HAV-positive serum specimens. These peptides immunoreacted with more than 30.0% of tested serum specimens with a range from 32.6 to 56.1%. All three peptides contain antigenic epitopes that have not been previously reported. Overlapping peptides, 1215 and 1216, demonstrated approximately a two times higher background immunoreactivity with anti-HAV-negative serum specimens (data not shown). Also, peptide 1216 demonstrated nonspecific immunoreactivity with 4 of 46 anti-HAV negative specimens (Table 1). Thus, the region at position 110–143 aa, as modeled with two consecutive overlapping synthetic peptides, exhibited HAV nonspecific reactivity under the experimental conditions that were uniformly applied to all peptides (see Materials and Methods).

Several peptides comprising regions 65–85 aa (Chikin *et al.*, 1991), 92–122 aa (Kulik *et al.*, 1994, 1995), 103–122 aa (Kulik *et al.*, 1994), and 119–130 aa (Bosch *et al.*, 1998) from the HAV VP2 protein and region 1–23 aa from the HAV VP4 protein have been synthesized and tested with anti-HAV-positive serum specimens previously. Among these peptides, only one, containing sequence 65–85 aa, was found to be not immunoreactive (Chikin *et al.*, 1991). However, this peptide significantly overlaps with the strongly immunoreactive peptide 1211, which contains this sequence (Table 1). The overlapping peptides 92–122 (Kulik *et al.*, 1994, 1995)and 103–122 aa (Kulik *et al.*, 1994) were found immunoreactive with convalescent-phase serum specimens and monoclonal antibodies obtained against the whole virus. These two peptides share sequences with peptide 1214, which is not immunoreac-

FIG. 1. Antigenic reactivity of synthetic peptides. (A) HAV polyprotein; (B) antigenic domains; 1–6, most immunoreactive antigenic domains; (C) vertical bars show the location of the center of each synthesized peptide; (D) percentage of serum specimens that are immunoreactive with each peptide; shaded area shows the average percentage immunoreactivity for peptides derived from each protein and dotted line shows the average percentage immunoreactivity for all peptides; (E) average *P/N* ratio calculated for all serum specimens immunoreactive with each synthetic peptide, where *P* represents the OD_{492} of anti-HAV-positive specimens and *N* represents the OD_{492} of negative specimens; shaded area shows the average *P/N* value for peptides derived from each protein and the dotted line shows the average *P/N* for all peptides; (F) the A index for each peptide (see Materials and Methods); dark shaded area shows the average A index for peptides derived from each protein, and the light shaded area shows the value that is two times greater than the average A index for the entire polyprotein shown by dotted line; (G) predicted secondary structure (Chou, 1990); black bars, alpha-helix; shaded bars, beta-structure; small black bars, beta-turns; horizontal line, random coil; (H) hydropathic plot (Kyte and Doolittle, 1982); hydrophilic regions are shown above the line.

TABLE 1

Antigenic Reactivity of Synthetic Peptides Derived from the HAV Structural Proteins

Peptide	Sequence	Position aa	Acute[1] sera	P/N[2] acute sera	NHS[3]
VP4-VP2					
YK-1206	GLDBILSLADIEEEQMIQSV	15-34	8/42 19.1%	9.46 (3.01-27.07)	0/46
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
YK-1210	EVGSBQVEPLRTSVDKPGSK	57-76	15/46 32.6%	7.31 (3.93-20.47)	0/48
YK-1211	EPLRTSVDKPGSKETQGEKP	64-83	21/46 45.7%	9.33 (3.37-27.92)	0/48
YK-1212	DKPGSKETQGEKPFLIESAD	71-90	3/41 7.3%	3.5 (3.04-3.96)	0/46
YK-1215*	[illegible]	110-129	10/41 24.4%	5.29 (3.31-8.63)	0/48
YK-1216*	[illegible]	124-143	29/41 70.7%	8.37 (3.32-10.79)	4/46
YK-1217*	[illegible]	137-156	2/41 4.9%	4.0 (3.94-4.12)	0/46
YK-1222	HFKDPQYPVWELTIRVWSEL	194-213	1/41 2.4%	4.29	0/46
YK-1224	[illegible]	214-233	9/41 22.0%	5.59 (3.09-8.17)	0/46
VP3					
YK-1235	SDPSQGGGDLKITHFTTWTSI	283-302	11/46 23.9%	4.2 (3.0-4.95)	0/48
YK-1236	GGDKITHFTTWTSIPTLAAQ	289-308	22/46 47.8%	4.68 (3.1-9.33)	0/48
YK-1210*	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
YK-1242	ENASDSVGDQQIKVIPVDPYF	311-330	1/41 2.4%	3.1	0/46
YK-1243	SDSVGDQIKVIPVDPYFQVM	314-333	1/41 2.4%	3.47	0/46
YK-1244	IKVIPVDPYFQMTNENPIDQ	321-340	6/41 14.6%	4.2 (3.55-5.2)	0/46
YK-1248	FWRGDLVFDFQVFPTKYHSG	355-374	2/41 7.3%	3.16; 3.2	0/46
YK-1249*	FDFQVFPTKYHSGRLLFCFV	362-381	18/46 39.1%	5.03 (3.07-7.03)	0/48
VP3					
YK-1250*	FPTKYHSGRLLFCFVPGNBL	367-386	17/46 37.0%	5.68 (3.4-7.97)	0/48
YK-1252	GIPLKQVATTAPGYVMDIDV	401-420	2/41 4.9%	4.65; 17.95	0/46
YK-1261*	VASHVRVNVYLSAINLBCPA-	461-480	16/41 39.0%	5.27 (3.72-7.9)	1/46
VP1					
YK-1265	TTVSTEQNVPDPQVGITTMK	501-520	9/41 22.0%	6.7 (3.25-9.57)	0/46
YK-1266	QNVPDPQVGITTMKDLKGKA	507-526	14/46 30.4%	8.25 (3.27-18.50)	0/48
YK-1268	NRGKMDVNQVQAPVGATTI	527-546	7/46 15.2%	9.1 (6.07-15.25)	2/48
YK-1271	ITTIEDPVLAKKVPETPPEL	543-562	4/41 9.8%	4.97 (3.33-7.76)	1/46
YK-1273	EDPVLAKKVPETPPELKPGE	547-566	6/41 14.6%	5.4 (4.87-6.12)	1/46
YK-1275*	AKKVPETPPELKPGESRETS	552-571	19/46 41.3%	5.15 (3.15-8.58)	3/48
YK-1276	PPELKPGESRETSDEMSTYK	559-578	9/46 19.6%	5.31 (3.0-8.47)	1/48
YK-1278	[illegible]	572-591	17/41 41.5%	4.48 (3.17-5.62)	0/46
YK-1279	HFLCTFTFNSNNKEYTFPIT	584-603	7/41 17.1%	3.43 (3.11-3.67)	1/48
YK-1280	TEVGLAVDTPEVESRLSLSI	654-673	6/48 12.5%	3.45 (3.0-4.11)	0/51
YK-1307	LSFSCYLEVTEQSEFYYFRA	734-753	6/48 12.5%	5.88 (4.17-7.64)	0/51
YK-1308	PLNSNAMLSTESVMSSSIAAG	754-773	2/48 4.2%	3.68; 5.29	0/51
YK-1312	MSRIAAGDLESSVDDPRSEB	767-786	13/48 27.1%	8.7 (4.44-25.9)	2/51
YK-1313	AGDLESSVDDPRSEEDKRFB	773-791	18/48 37.5%	15.79 (3.0-108.06)	3/51
YK-1314	VDDPRSEEDKRFESEIECRK	779-798	26/48 54.2%	14.68 (3.53-89.96)	1/51

Note. Alternating boldface and italic type represent individual antigenic domains.

[1] The number of positive serum samples divided by the total number of tested sera and percentage of immunoreactive sera.

[2] Mean *P/N* value; number in parentheses indicates the range of *P/N* values.

[3] Normal human sera.

[4] Background reactivity with anti-HAV-negative sera is two times greater than that for other peptides.

tive and includes the sequence 87–106 aa, and peptide 1215, which is immunoreactive and comprises the sequence 110–129 aa. It is conceivable that the overlapping region between these three immunoreactive peptides at position 110–122 aa may represent at least an essential part of a common antigenic epitope. The other immunoreactive peptide, described by Bosch *et al.* (1998) and comprising region 119–130 aa, essentially overlaps immunoreactive peptide 1215 containing the sequence 110–129 aa (Table 1). Thus, the common antigenic epitope modeled with these peptides may be located within the region at position 119–129 aa. It is interesting to note that the last region contains residue Tyr_{123}, which was found exposed on the surface of HAV particles (Robertson *et al.*, 1989). Peptide comprising the entire HAV VP4 protein (1–23 aa) has been synthesized and shown not to be immunoreactive with anti-HAV-positive convalescent-phase serum specimens (Chikin *et al.*, 1991). Two peptides that overlap this sequence were used in this study. One peptide comprising the sequence 1–19 aa was not immunoreactive, while the other peptide, 1208, was immunoreactive (Table 1). Since the peptide containing the region 1–23 aa was tested by using convalescent-phase serum specimens, which are, in general, less immunoreactive than the acute specimens used in the present study, it is impossible to determine

whether the VP4 region at position 15–23 aa, as represented within peptide 1206 (Table 1), contains an antigenic epitope or the antigenic reactivity is attributed entirely to the N-terminal part (24–34 aa) of the VP2 protein included within this peptide.

VP3 protein. Among 37 peptides spanning the entire HAV VP3 protein, 11 peptides were found to be immunoreactive (Fig. 1). An analysis of overlapping immunoreactive peptides allowed the identification of five antigenic domains within this protein (Table 1). Two C-terminal domains of the VP3 protein were identified with one peptide each, while the N-terminal domain was identified with two overlapping peptides. Two central domains were identified with three and four immunoreactive synthetic peptides (Table 1), respectively. Four peptides (1236, 1249, 1250, and 1261) that belong to three different antigenic domains were found to be broadly immunoreactive. These peptides demonstrated immunoreactivity with more than 30.0% of anti-HAV-positive serum specimens. Therefore, three domains at positions 283–308, 355–386, and 461–480 aa also contain the most immunoreactive epitopes, as modeled with synthetic peptides. Three peptides derived from regions at positions 362–386 and 461–480 aa demonstrated high background immunoreactivity (Table 1). Peptide 1261 demonstrated nonspecific immunoreactivity with 1 of 48 anti-HAV-negative specimens under the experimental conditions that were uniformly applied to all synthetic peptides (see Materials and Methods).

Peptides derived from regions at positions 290–302 aa (Kulik *et al.*, 1994), 321–330 aa (Chikin *et al.*, 1991), 355–366 aa (Bosch *et al.*, 1998), and 382–395 aa (Kulik *et al.*, 1994, 1995) were previously tested for antigenic reactivity with human anti-HAV-positive convalescent-phase serum specimens and with monoclonal antibodies. Except for peptide containing the sequence 321–330 aa, three of these peptides were found to be immunoreactive. It is interesting that the sequence of the nonimmunoreactive peptide was represented within two immunoreactive overlapping peptides, 1243 (314–333 aa) and 1244 (321–340 aa), described in the present study (Table 1). Conversely, the peptide containing the sequence 382–395 aa has been previously reported as immunoreactive (Kulik *et al.*, 1994, 1995); however, peptide 1251 (380–399 aa), which contains this complete sequence, was not found to be immunoreactive in our study (Table 1). Peptide 1250 (367–386 aa) and 1252 (391–410 aa) are partially overlapped with an antigenic epitope at position 382–395 aa previously identified by Kulik *et al.* (1995). However, these peptides do not appear to model this epitope because neither peptide contains the complete sequence of this epitope. The antigenic reactivity of two other peptides containing sequences at positions 290–302 aa (Kulik *et al.*, 1994) and 355–366 aa (Bosch *et al.*, 1998) was confirmed in the present study with peptides 1235 (283–302 aa) and 1248 (355–374 aa) (Table 1).

Three weakly immunoreactive peptides (1241, 1242, and 1243) contain residues Asp_{318} and Gln_{319}, which have been found to be essential residues of the HAV major conformational neutralizing epitope (Lemon, 1992; Ping *et al.*, 1988; Ping and Lemon, 1992). These peptides also contain the region at position 308–321 aa, known to elicit antibody that recognizes HAV particles (Lemon, 1992).

VP1 protein. Among 52 synthetic peptides spanning the entire HAV VP1 protein, 15 were found to be anti-HAV immunoreactive (Fig. 1). These peptides identify nine antigenic domains (Table 1). Six domains at positions 527–546, 572–591, 584–603, 651–670, 734–753, and 754–773 aa were identified with only one peptide each. The N-terminal domain at position 501–526 aa was identified with 2 peptides, while the C-terminal domain at position 767–798 aa was identified with 3 peptides. One domain at position 543–578 aa was identified with 4 peptides (Table 1). Five peptides (1266, 1273, 1276, 1313, and 1314) derived from four antigenic domains were found to be immunoreactive with more than 30.0% of anti-HAV-positive serum specimens. However, 2 of these broadly immunoreactive peptides demonstrated a two times higher background reactivity with anti-HAV-negative specimens compared to all the other peptides (Table 1). Moreover, 1 of these 2 peptides, 1273, was shown to immunoreact with 3 of 48 anti-HAV-negative specimens, thus demonstrating nonspecific reactivity. Another broadly immunoreactive peptide, 1313, also demonstrated nonspecific reactivity with about 6.0% of anti-HAV-negative serum specimens (Table 1). It should be noted that 10 of 15 VP1-derived immunoreactive peptides were shown to exhibit HAV nonspecific antigenic reactivity compared to only 2 peptides among all peptides derived from both VP2 and VP3 proteins (Table 1). It should be emphasized, however, that the assay experimental conditions were uniformly applied to all synthetic peptides without regard to optimizing assay conditions for each peptide (see Materials and Methods). It is likely that with proper optimization, especially for the concentration of each peptide adsorbed to microtiter wells, the nonspecific immunoreactivity might be significantly reduced or eliminated.

A number of peptides derived from the HAV VP1 protein were previously tested for antigenic reactivity. Peptide containing the sequence at position 502–516 aa was shown to elicit HAV neutralizing antibodies (Bosch *et al.*, 1998; Emini *et al.*, 1985; Lemon *et al.*, 1992). This peptide was also recently found to be immunoreactive with anti-HAV antibodies (Bosch *et al.*, 1998). This finding contradicts a previous study in which no antigenic reactivity was found associated with a peptide of the same sequence (Kulik *et al.*, 1994, 1995). In our experiments, the peptide 1265, completely comprising the sequence of this peptide, was shown to immunoreact with 22.0% of acute-phase anti-HAV-positive serum specimens (Table 1), thereby confirming the presence of an antigenically

reactive epitope within this region of the HAV VP1 protein.

Another antigenic region within the HAV VP1 protein was previously found by using a set of three peptides containing the sequence 606–630 aa (Kulik *et al.*, 1994, 1995). However, this observation was not confirmed by results obtained with peptide 609–631 aa (Chikin *et al.*, 1991), which was found not to be immunoreactive. In our study, we also could not confirm the presence of an antigenic epitope(s) within this region, since peptide 1284 containing the sequence 606–625 aa did not show any HAV-specific antigenic reactivity.

Additionally, a set of peptides derived from regions at position 492–508, 564–577, 589–599, 599–614, and 767–789 aa was previously tested with anti-HAV-positive serum specimens (Chikin *et al.*, 1991). None of these peptides, however, was found to be immunoreactive. In this study, two peptides, 1264 (490–509 aa) and 1283 (598–617 aa), comprising two of these regions, were also nonimmunoreactive, thus confirming the absence of antigenic reactivity within regions 492–508 and 599–614 aa. However, peptide 1274 (559–578 aa), 1279 (584–603 aa), 1312 (767–786 aa), and 1313 (772–791 aa), which completely comprised sequences from the other three regions, were found to be immunoreactive, with peptide 1313 demonstrating the strongest and broadest immunoreactivity (Table 1). Interestingly, immunoreactive peptide 1279 contains Ser_{102}, which was shown to be an important residue of the major HAV neutralizing antigenic epitope (Lemon, 1992; Ping *et al.*, 1988; Ping and Lemon, 1992). Immunoreactive peptide 1290 (Table 1) contains another two amino acids, Val_{171} and Ala_{176}, also shown to be essential for the activity of this neutralizing epitope (Lemon, 1992; Ping *et al.*, 1988; Ping and Lemon, 1992).

Peptides 1304, 1305, 1306, and 1307 are located within the region at position 714–752 aa that was previously shown to be exposed on the surface of HAV particles (Robertson *et al.*, 1989). Surface exposure is generally considered to be an important indicator of potential antigenic reactivity (Emini *et al.*, 1985); however, among these peptides, only peptide 1307 was found to be immunoreactive (Table 1).

Immunoreactivity of peptides derived from nonstructural proteins

The antigenic composition of the entire HAV nonstructural area has not been previously reported. It is known, however, that the nonstructural proteins are antigenically reactive and may bind antibodies from anti-HAV-positive serum specimens (Jia *et al.*, 1992; Robertson *et al.*, 1992, 1993; Steward *et al.*, 1997). Nevertheless, no attempt has been made to map antigenic epitopes within these proteins.

P2A protein. Five peptides, 1315, 1316, 1317, 1318, and 1319, were synthesized to span the entire P2A protein (Fig. 1). Among these peptides, four were found to be immunoreactive (Table 2). All antigenically active P2A peptides could bind antibodies from 31.3 to 83.3% of acute-phase anti-HAV-positive serum specimens with average *P/N* values varying from 5.94 to 19.35 (Table 2). Peptide 1317 immunoreacted with 83.3% of acute-phase serum specimens and as such represents the most immunoreactive peptide found in the present study. Thus, the HAV nonstructural P2A protein is very antigenically reactive (Fig. 1). However, the P2A antigenic domain should be considered an extension to the C-terminal antigenic domain of the HAV VP1 protein since all overlapping consecutive peptides from 1312 to 1318 were strongly immunoreactive (Tables 1 and 2). Thus, the region spanned by these peptides at position 767–842 aa that comprise the C-terminal sequence of the VP1 protein and almost the entire P2A protein may be considered as one immunodominant domain of the HAV polyprotein (Fig. 1B, region 2).

P2B protein. Among 18 peptides derived from the HAV P2B protein, only 2 were found to be immunoreactive (Figs. 1D and 1E). These immunoreactive peptides identified two small independent antigenic domains at positions 922–941 and 961–980 aa (Table 2). Both peptides were very immunoreactive as evidenced by each peptide reacting with approximately 30.0% of acute-phase HAV serum specimens. The HAV P2B protein is the least immunoreactive region compared to all other HAV proteins, as assessed by the antigenic reactivity of synthetic peptides.

P2C protein. Among 30 peptides spanning this protein, 10 were found to be immunoreactive (Figs. 1D and 1E). These immunoreactive peptides identified six antigenic domains (Table 2). The N-terminal and the C-terminal domains at positions 1133–1152 and 1403–1422 aa, respectively, were identified with only one peptide each. Two internal domains at positions 1261–1280 and 1299–1318 aa were also identified with one peptide each. One domain at position 1210–1239 aa was identified with two peptides, while the domain at position 1331–1379 aa was spanned by four immunoreactive peptides (Table 2). None of the HAV P2C peptides was immunoreactive with more than 30.0% serum specimens. The most immunoreactive peptides, 1347, 1348, and 1367, were found to be immunoreactive with only 22.0–29.2% of sera (Table 2).

P3A protein. This protein contains only one antigenic domain (Table 2), located at the N-terminal region (Fig. 1B). Among six peptides spanning this protein, three were found to be immunoreactive; two of these peptides, 1368 and 1369, demonstrated strong immunoreactivity (Table 2). Consistent with the definition of an antigenic domain as presented in this paper, the P3A antigenic domain should be extended to include the C-terminus of the P2C protein, where immunoreactive peptide 1367 from the P2C protein is consecutive with peptide 1368 from the P3A protein (Table 2). Thus, as observed for the

TABLE 2

Antigenic Reactivity of Synthetic Peptides Derived from the HAV Nonstructural Proteins

Peptide	Sequence	Position aa	Acute[1] sera	P/N[2] acute sera	NHS[3]
P2A					
YK-1315	SEIECRKPYKELRLEVCKQR	792-811	26/48 54.2%	13.71 (3.1-75.61)	1/51
YK-1316	PYKELRLEVGKQRLKYAQEE	799-818	20/48 41.7%	8.71 (3.5-17.8)	2/51
YK-1317	QRLKYAQEELSNEVLPPPRK	810-829	55/66 83.3%	19.35 (3.47-125.8)	1/66
YK-1318	VLPPPRKMKGLFSQAKISLF	823-842	15/48 31.3%	5.94 (3.4-8.68)	0/51
P2B					
YK-1327	KVNEFHGMLDLEELAANSKD	922-941	45/48 [illegible]	6.16 (3.28-9.76)	1/51
YK-1331	KINLADRMLGLSGVQEIKEQ	961-980	13/48 27.1%	5.71 (3.71-10.3)	0/51
P2C					
YK-1341	NILKDNQQKIEKAIEEADEF	1133-1152	3/48 6.3%	5.23 (3.44-8.26)	1/51
YK-1347	LGSINQAMVTRCEPVVCYLY	1210-1229	13/48 27.1%	4.16 (3.16-6.2)	2/51
YK-1348	RCEPVVCYLYGKRGGGKSLT	1220-1239	14/48 29.2%	6.96 (3.64-15.48)	2/51
YK-1352	TKPVASDYWDGYSGQLVCII	1261-1280	8/48 16.7%	4.45 (3.38-5.81)	0/51
YK-1356	VSGCPMRLNMASLEEKGRHF	1299-1318	8/48 16.7%	5.49 (3.56-10.96)	0/51
YK-1360	NPSPKTVYVKEAIDRRLHFK	1331-1350	4/41 9.8%	4.08 (3.68-4.71)	0/46
YK-1361	VKEAIDRRLHFKVEVKPASF	1339-1358	5/41 12.2%	4.12 (3.21-6.28)	0/46
YK-1362	VKPASFFKNPHNDMLNVNLA	1353-1372	6/41 14.6%	7.82 (3.4-18.05)	0/41
YK-1363	KNPHNDMLNVNLAKTNDAIK	1360-1379	5/41 12.2%	5.96 (5.31-6.33)	0/41
YK-1367	VMTVEIRKQNMTEPMELWSQ	1403-1422	9/41 22.0%	7.02 (4.18-14.26)	0/41
P3A					
YK-1368	SQGISDDDNDSAVAEFFQSF	1421-1440	26/41 63.4%	9.58 (4.27-19.82)	1/27
YK-1369	DSAVAEFFQSFPSGEPSNSK	1430-1449	24/41 58.5%	21.71 (3.8-42.63)	1/27
YK-1370	FQSFPSGEPSNSKLSGFFQS	1437-1456	10/41 24.4%	8.05 (3.05-18.48)	1/27
P3B					
YK-1374	EGVTKFKQVIKLDADPVESQ	1500-1519	18/41 43.9%	18.57 (3.28-29.07)	0/27
P3C					
YK-1376	GLVRKNLVQFGVGEKNGCVR	1526-1545	3/41 7.3%	6.71 (3.8-12.77)	0/27
YK-1382	DVVLMKVPTIPKFRDITQHF	1603-1622	1/41 2.4%	8.4	0/27
YK-1388	MEEKATYVEKKNDGTTVDLT	1656-1675	4/41 9.8%	4.29 (3.99-4.7)	0/27
YK-1389	KNDGTTVDLTVDQAWRGKGE	1666-1685	3/41 7.3%	10.33 (5.53-18.26)	0/27
YK-1390	RGKGEGLPGMCGGALVSSNQ	1681-1700	10/41 24.4%	6.62 (3.52-13.98)	1/27
YK-1393	VAKLVTQEMFQNIDKKIESQ	1719-1738	24/41 58.5%	10.45 (4.64-16.36)	1/27
P3D					
YK-1394	RIMKVEFTQCSMNVVSKTLF	1739-1758	13/41 31.7%	6.98 (4.05-12.93)	3/27
YK-1395	FTQCSMNVVSKTLFRKSPIY	1745-1764	8/41 19.5%	6.52 (3.51-17.52)	1/27
YK-1399	MLSKYSLPIVEEPEDYKEAS	1791-1810	5/41 12.2%	6.86 (4.05-16.46)	1/27
YK-1407	LDENGLLLGVHPRLAQRILF	1866-1885	2/41 4.9%	3.43; 3.98	0/27
YK-1411	CPKDELRELEKVLESKTRAL	1903-1922	8/41 19.5%	8.18 (4.65-17.65)	0/27
YK-1412	SKTKAIDACPLDYSILCRMY	1917-1936	10/41 24.4%	6.04 (3.4-15.15)	2/27
YK-1418	KTMIRFGDVGLDLDFSAFDA	1969-1988	6/41 14.6%	4.5 (3.35-6.21)	1/27
YK-1419	DLDFSAFDASLSPFMIREAC	1980-1999	5/41 12.2%	5.27 (3.98-8.49)	1/27
YK-1424	INNVNLYYVESKIFGKSPVF	2052-2071	3/41 7.3%	6.69 (3.48-10.53)	0/27
YK-1429	LGMTATEADKNVPQLKFVSE	2112-2131	5/41 12.2%	10.25 (4.92-21.38)	0/27
YK-1434	SEKTIWSLIAWQRSNAEFEQ	2151-2170	11/41 26.8%	7.47 (3.85-16.03)	1/27
YK-1435	SLIAWQRSNAEFEQNLENAQ	2157-2176	6/41 14.6%	8.96 (5.16-13.71)	1/27
YK-1436	WQRSNAEFEQNLENAQWEAF	2161-2180	4/41 9.8%	4.49 (3.55-5.09)	1/27

Note. Alternating boldface and italic type represent individual antigenic domains.
[1] The number of positive serum samples divided by the total number of tested sera and percentage of immunoreactive sera.
[2] Mean *P/N* value; number in parentheses indicates the range of *P/N* values.
[3] Normal human sera.

P2A protein, the P3A protein contains one very strong antigenic domain that extends into the C-terminus of the preceding protein.

P3B protein. This short 23-aa protein was spanned by only one peptide, 1374 (Fig. 1), which demonstrated very broad and strong antigenic reactivity (Table 2). This peptide and those derived from P2A and P3A proteins represent the most diagnostically relevant peptides identified in this study.

P3C protein. Among 19 peptides synthesized spanning the entire P3C protein, 6 were immunoreactive (Figs. 1D and 1E). One peptide, 1393, derived from the very C-terminus was found to be one of the most immunoreactive peptides described in this study (Table 2) as evidenced by its reacting with almost 60.0% of acute-phase anti-HAV-positive serum specimens. The HAV P3C protein contains four antigenic domains, three of which were identified with one peptide each.

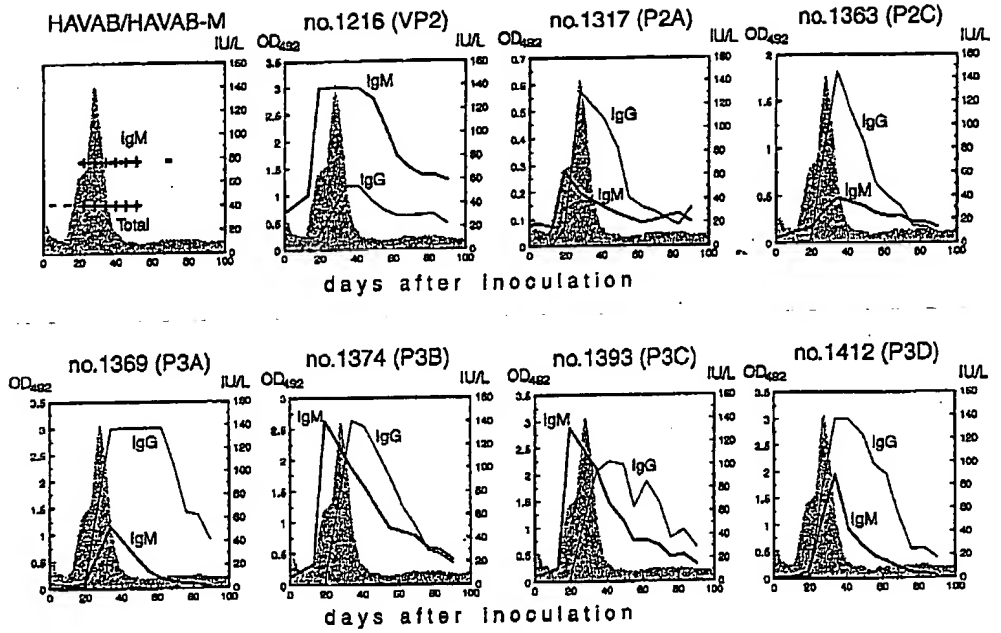

FIG. 2. IgG and IgM anti-HAV immunoreactivity of synthetic peptides with a seroconversion panel obtained from an experimentally HAV-infected chimpanzee 1357. Shaded area shows the ALT level. HAVAB/HAVAB-M panel shows the results for the detection of IgM and total antibodies with HAVAB-M and HAVAB diagnostic tests, respectively; plus and minus signs indicate positive and negative results of testing; the other specimens were not tested.

One domain was identified with three peptides (Table 2).

P3D protein. Forty-eight peptides were derived from this protein; 13 were found to be immunoreactive (Fig. 1). These immunoreactive peptides identified eight antigenic domains (Table 2). Four domains were identified with one peptide each, three were identified with 2 peptides each, and one was identified with 3 peptides (Table 2). Peptide 1394 was the most immunoreactive among all the P3D peptides. This peptide immunoreacted with approximately 32.0% of anti-HAV-positive acute serum specimens (Table 2). Similar to the P2A and P3A proteins, the P3D N-terminal antigenic domain may be extended into the C-terminal region of the P3C protein (Table 2).

Immunoreactivity of peptides with HAV seroconversion panels

All synthetic peptides presented in Tables 1 and 2 were tested with seven anti-HAV seroconversion panels obtained from HAV-infected patients ($n = 2$) and from experimentally infected chimpanzees ($n = 5$; see Materials and Methods). A specific seroconversion pattern of antibody binding was detected for all peptides. This finding demonstrates that the peptides shown in Table 1 and 2 specifically detected anti-HAV antibodies. The majority of the peptides demonstrated both IgG and IgM anti-HAV activity. A few examples of specific reactivity for representative peptides from each HAV protein, with follow-up serum specimens obtained from experimentally infected chimpanzee 1357 and with seroconversion panel RP-004 obtained from an HAV-infected patient, are shown in Figs. 2 and 3, respectively. It is interesting to note that IgM antibody can be detected with synthetic peptides for approximately the same period of time as with cell culture-derived antigen used in the commercially available test (HAVAB-M, Abbott Laboratories), whereas IgG anti-HAV activity can be detected for a much shorter period compared to HAVAB (Figs. 2 and 3). This observation may be explained by a relatively lower immunoreactivity with convalescent-phase anti-HAV-positive serum samples of even strongly immunoreactive peptides such as peptide 1317 (data not shown) in concordance with previous observations using synthetic peptides and convalescent-phase serum specimens (Bosch *et al.*, 1998; Chikin *et al.*, 1991; Kulik *et al.*, 1994, 1995).

Almost all synthetic peptides demonstrated a uni-

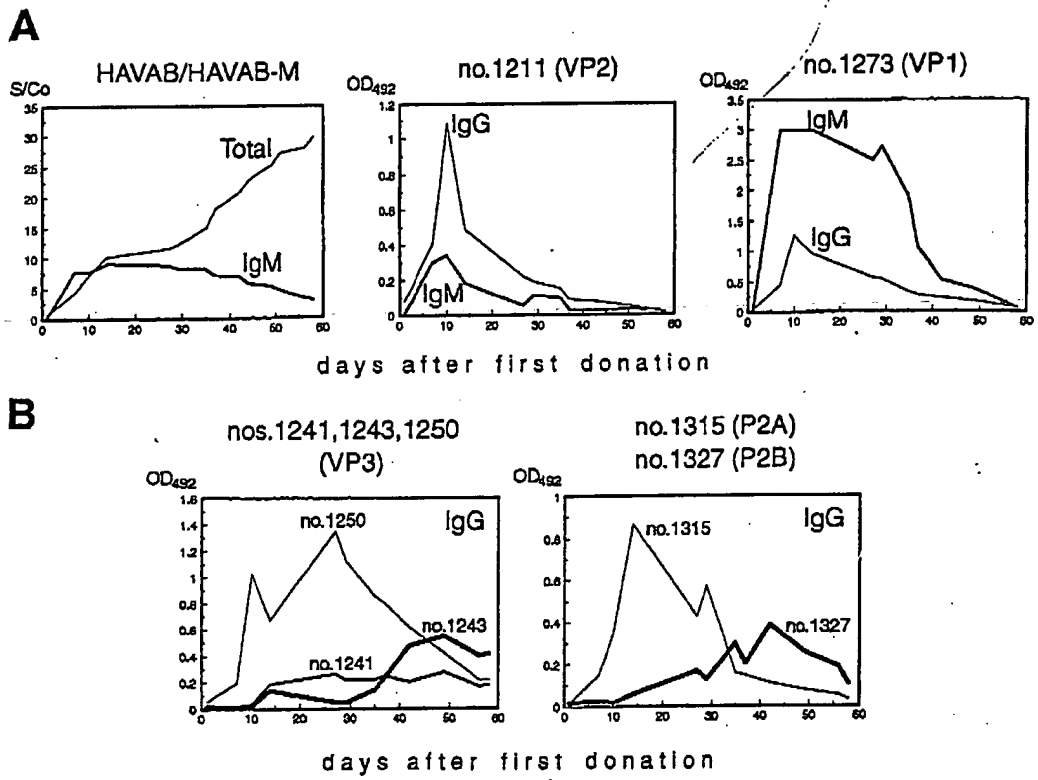

FIG. 3. IgG and IgM anti-HAV immunoreactivity of synthetic peptides with the seroconversion panel RP-004. HAVAB/HAVAB-M panel shows results for the detection of IgM and total antibodies with HAVAB-M and HAVAB diagnostic tests, respectively.

form pattern of immunoreactivity with the various seroconversion panels, as can be seen in Figs. 2 and 3A. Some peptides, however, showed a slightly different pattern of immunoreactivity (Fig. 3B). For example, peptides 1250 and 1315 (Fig. 3B) showed a typical reactivity with the seroconversion panel RP-004, which is very similar to the patterns seen with other peptides in Figs. 2 and 3A; by contrast, peptides 1243 and 1327 showed a "delayed" immunoreactivity pattern. It is interesting to note that peptides 1241, 1243, and 1250, which all belong to the VP3 protein, demonstrated two distinct patterns of immunoreactivity, typical and delayed (Fig. 3B).

Antigenic composition of the HAV polyprotein

In the present study a number of antigenic epitopes were identified by using synthetic peptides (Tables 1 and 2). These antigenic epitopes were modeled with 75 peptides almost uniformly scattered across the entire HAV polyprotein (Fig. 1). As mentioned above, some antigenic epitopes from the structural proteins had been previously discovered (Bosch *et al.*, 1998; Emini *et al.*, 1985; Kulik *et al.*, 1994, 1995), whereas none of the nonstructural antigenic epitopes had been previously identified. The finding of strong antigenic epitopes within the nonstructural proteins corroborates previous observations that antibodies specific to nonstructural proteins may be detected in anti-HAV-positive serum samples with protein products expressed in an *in vitro* transcription–translation system (Jia *et al.*, 1992; Robertson *et al.*, 1992, 1993; Stewart *et al.*, 1997).

By the use of synthetic peptides, 40 antigenic domains were found scattered along the entire polyprotein, and antigenic epitopes were identified within every HAV protein. An assessment of the antigenic reactivity of peptides reveals that, in general, structural proteins showed an average antigenic reactivity, whereas nonstructural proteins such as P2A, P3A, and P3B are very antigeni-

cally reactive (Figs. 1D, 1E, and 1F). Protein P2B, however, is the least immunoreactive (Fig. 1D). Approximately 30 of 75 immunoreactive peptides demonstrated a percentage immunoreactivity (Fig. 1D) and an average *P/N* value (Fig. 1E) greater than the average value calculated for all immunoreactive peptides. Only 13 peptides (1211 from VP2, 1266, 1312, 1313, and 1314 from VP1, 1315, 1316, and 1317 from P2A, 1368, 1369, and 1370 from P3A, 1374 from P3B, and 1393 from P3C) were found to exceed the average level for both parameters (Figs. 1D and 1E). Among these 13 peptides, only 9 (1211, 1313, 1314, 1315, 1317, 1368, 1369, 1374, and 1393) were identified with an antigenic index (see Materials and Methods) that was two times greater than the average value for the entire polyprotein (Fig. 1F). These 9 peptides belong to five antigenic domains (Fig. 1B). Domain 1, which is the only structural domain among the five strong antigenic domains, is located at position 57–90 aa. Domain 2, at position 767–842 aa, encompasses the C-terminal region of the VP1 protein and the entire P2A protein. Domain 3 contains the C-terminal region of the P2C protein and the N-terminal region of the P3A protein at position 1403–1456 aa. Domain 4 contains almost the entire P3B protein at position 1500–1519 aa. Domain 5 contains the C-terminal region of the P3C protein and the N-terminal region of the P3D protein at position 1719–1764 aa (Fig. 1B, Tables 1 and 2). It is interesting to note that three of the five strong antigenic domains such as domains 2, 3, and 5 contain the predicted protease cleavage sites (Fig. 1B). Another interesting observation is that domains 2, 3, and 4 belong to the smallest HAV proteins, P2A, P3A, and P3B. All five strong antigenic domains are hydrophilic (Fig. 1H) and are folded into alpha-helices separated by strong beta-turns (Fig. 1G), as predicted using computer-assisted analysis of secondary structure (Chou, 1990). Domain 2, the most immunoreactive domain, comprises the most hydrophilic region, which is also part of the largest predicted alpha-helical region within the HAV polyprotein (Figs. 1G and 1H).

An analysis of the immunoreactivity of synthetic peptides with HAV seroconversion panels demonstrated that both IgM and IgG antibodies can be detected with synthetic peptides for a short period of time around the acute phase of HAV infections (Figs. 2 and 3). A similar pattern of reactivity was found for peptides derived from both structural and nonstructural proteins. This finding suggests that several HAV-specific antigenic epitopes, which can be efficiently modeled with synthetic peptides, elicit antibodies for only a short time. Alternatively, synthetic peptides, because of some innate property such as low avidity of antibody binding, can efficiently detect anti-HAV antibodies only during the acute phase of infection. Recently, a similar short-term antibody response was observed with a recombinant P3C protein (Stewart *et al.*, 1997). This observation, taken together with our findings, strongly suggests that the immune response to the HAV nonstructural proteins is usually of short duration. Nevertheless, synthetic peptides or antigenic epitopes identified in the present study may have potential diagnostic application, especially for the detection of acute HAV infections currently diagnosed by the detection of IgM anti-HAV using cell-culture-derived virus as the antigenic target. Recombinant proteins or synthetic peptides from the structural proteins have not been successfully used as alternative sources of antigen in the development of enzyme immunoassays for the detection of anti-HAV because of apparently poor antigenic reactivity. Poor performance of these antigens has been explained by the strictly conformational nature of HAV antigenic epitopes (Lemon, 1992). In addition, current commercially available assays are unable to discriminate between natural infections and vaccine-induced immunity (Jia *et al.*, 1992; Robertson *et al.*, 1992, 1993; Stewart *et al.*, 1997). Antigenic domains discovered within the HAV nonstructural proteins may be most suitable for developing such an assay. Finally, data derived in the present study may be used to construct recombinant proteins or artificial antigens similar to the mosaic hepatitis E antigen (Khudyakov *et al.*, 1994) and used as antigenic targets in the development of diagnostic tests for the detection of acute HAV infections.

MATERIALS AND METHODS

Synthetic peptides

Peptides were synthesized by FMOC chemistry (Barany and Merrifield, 1980) on an ACT Model MPS 350 multiple peptide synthesizer (Advanced Chemtech, Louisville, KY) according to the manufacturer's protocols. After characterization by amino acid analysis, high-performance liquid chromatography, and capillary electrophoresis, peptides were characterized by enzyme immunoassay.

Serum samples

All anti-HAV-positive serum samples collected from anti-HAV-positive patients with acute liver disease and anti-HAV-negative serum samples collected from normal blood donors were randomly selected from a collection reposited at the Centers for Disease Control and Prevention (Atlanta, GA). All serum specimens were initially tested by commercially available kits for the presence of IgG and IgM anti-HAV activity (HAVAB and HAVAB-M, Abbott Laboratories, North Chicago, IL).

Enzyme immunoassay for anti-HAV

Synthetic peptides (110 μl) at a concentration of 10 μg/ml in 0.1 M phosphate-buffered saline (PBS), pH 7.5, were adsorbed to microtiter wells (Immulon II, Dynatech Industries, Inc., McLean, VA) at room temperature for 12 h. Serum specimens were diluted in PBS containing

0.1% Tween 20 and 10% normal goat serum (PBS-T); 100 μl of diluted specimens was added to each well, incubated for 60 min at 37°C, and washed with PBS containing Tween 20. Two different dilutions of specimen and detector antibodies were used. One set of serum specimens was diluted 1:100. The antibody binding was detected by adding 100 μl of affinity-purified anti-human IgG coupled to horseradish peroxidase (HRP) (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1:30,000 and by incubating it for 1 h at 37°C. The second set of serum specimens was diluted 1:1000. In this case, 50 μl of affinity-purified anti-human IgG conjugated to biotin diluted 1:8000 and 50 μl of streptavidin coupled to HRP were added to microtiter wells and incubated for 30 min at 37°C. Two different cut-offs were established to identify anti-HAV specific immunoreactivity. The first cut-off, expressed as a *P/N* ratio and equal to 3.0, was statistically established as the mean of negative controls plus at least 3.5 standard deviations (SD) above the mean, where *P* represents the optical density value at 493 nm (OD_{493}) of anti-HAV-positive specimens and *N* represents the OD value of negative controls. An additional cut-off was used to ensure statistical reliability and accurate interpretation of positive results. In this case, in addition to anti-HAV-negative serum, two irrelevant synthetic peptides were used as supplemental negative controls to determine the degree of nonspecific binding of anti-HAV serum to synthetic peptides. One cut-off, expressed as a *P/N* ratio and equal to 3.0, corresponded to the mean of negative serum controls plus at least 3.5 SD above the mean. A second cut-off was also expressed as a *P/N* ratio, where *P* represents the OD value at 493 nm (OD_{493}) for HAV peptides immunoreacting with anti-HAV-positive specimens and *N* represents the OD value obtained with the irrelevant peptides. The second cut-off was equal to 2.0. Synthetic peptides were considered immunoreactive with anti-HAV serum specimens only when both criteria were satisfied. Tables 1 and 2 and Fig. 1 show *P/N* values obtained by using the anti-HAV-negative serum specimens.

The antigenic index, designated the A index (Fig. 1F), was derived by multiplying the mean of *P/N* values and percentage of serum specimens immunoreactive with each synthetic peptide (Tables 1 and 2) and therefore represents a measure of both the strength and the breadth of the immunoreactivity of each peptide with serum specimens. When antigenic properties of different peptides are compared, the A index can be calculated as the mean of *P/N* values for all anti-HAV-positive serum specimens tested with each peptide. Thus, the A index combines such parameters as the mean of *P/N* values of specimens tested positive with each peptide and the percentage of serum specimens immunoreactive with each peptide. In this study, because the mean of *P/N* values for specimens immunoreactive with each peptide and the percentage of immunoreactive sera were calculated long before, the A index was calculated as described above. This calculation gives different absolute value without affecting the relative value of the A index calculated as the mean of *P/N* in rating antigenic quality of synthetic peptides. We found that the A index in combination with such parameters as the average *P/N* of positive specimens and the percentage of immunoreactive serum specimens (Figs. 1D, 1E, and 1F) is a very helpful indicator of antigenic potential of peptides and as such was used in this study to measure the diagnostic relevance of each individual peptide.

HAV seroconversion panels

Two anti-HAV seroconversion panels, RP-004 and RP-013 (Profile Diagnostics, Inc., Sherman Oaks, CA), were used in this study. Panel RP-004 contains 15 members collected over 83 days from a patient with symptoms of hepatitis A infection including jaundice, malaise, dehydration, and elevated levels of alanine aminotransferase (ALT). Panel RP-013 also contains 15 members collected over 189 days from a patient with symptoms of hepatitis A infection.

Five seroconversion panels were obtained from experimentally HAV-infected chimpanzees (chimps 1357, 1402, 1439, 1487, and 1489). Chimp 1357 was injected intravenously with 10^8 chimpanzee infectious doses of wild-type HLD-2 HAV inoculum. Chimp 1402 was inoculated intravenously with 25 ng of live, attenuated virus (HAS-15, 160S fraction after sucrose gradient purification) without adjuvant, which resulted in no ALT elevation and no seroconversion. This animal was challenged with 1 ml wild-type virus (HLD-2) inoculated intravenously 218 days after the first inoculation. Chimps 1487 and 1489 were inoculated via an oral gastric tube with 1 ml SD-11 human stool suspension in veal broth (kindly provided by Dr. R. H. Purcell) containing 100 chimpanzee oral infectious doses. Chimp 1439 was inoculated intragastrically with a 10% stool suspension obtained from an experimentally HAV-infected cynomogus macaque (145), which resulted in no anti-HAV seroconversion and no ALT elevation, and was subsequently challenged with 1 ml wild-type virus (HLD-2) inoculated intravenously 145 days after the first inoculation. All animals exhibited ALT elevations and seroconverted following inoculation with HAV HLD-2. Twelve follow-up serum specimens collected from each animal over ~100 days after the inoculation were used to test the immunoreactivity of synthetic peptides in the present study.

Computer-assisted analysis

Amino acid sequence analysis was performed by using the Protean program from the Lasergene software package (DNASTAR Inc., Madison, WI). Hydropathic plots were built by the Kyte and Doolittle (1982) method

and protein secondary structure was predicted by the Chou and Fasman method (see Chou, 1990).

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EXHIBIT "B"

Serological approaches to distinguish immune response to hepatitis A vaccine and natural infection

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Currently, the immune status of an individual exposed to hepatitis A virus (HAV) is determined by assays which measure antibodies against the capsid proteins. These assays indicate exposure to the viral capsid that could result from either infection or from vaccination. Recent data indicate that proteins from the non-structural genome region of the virus (P2 or P3), which are only produced during active virus replication, generate antibodies after clinical disease. A sub-genomic cDNA segment of HAV corresponding to the P2 region was used for in vitro *transcription-translation followed by immune precipitation of the translated products under non-denaturing conditions. Serial serum specimens from experimentally infected chimpanzees and humans naturally infected with hepatitis A verified the development of antibodies to P2 proteins following infection. A serosurvey of individuals positive for antibodies to the HAV capsid (HAVAB assay, Abbott Laboratories) revealed that 50–60% of children and 16–32% of adults had no detectable antibodies to the P2 antigen by immune precipitation. These results may reflect subclinical infections resulting in a lower level of antibodies against the non-structural antigens or may represent a greater sensitivity of the competitive assay (HAVAB) used to detect capsid antibodies compared to the immunoprecipitation assay used to detect non-structural antigens.*

Keywords: HAV; structural and non-structural antigen; *in vitro* transcription/translation

INTRODUCTION

Currently, diagnosis of acute infection or prior exposure to hepatitis A virus (HAV) is based on the detection of immunonoglobulin M (IgM) or IgG antibodies to capsid proteins, respectively. The presence of IgM antibodies is used to diagnose asymptomatic, subclinical or clinical cases of hepatitis A while IgG antibodies are used to evaluate the immunity to HAV infection in selected cases. The IgM antibody population can generally be detected for 3–6 months after infection; however, an IgG antibody response to the HAV capsid can be detected indefinitely and is currently used as an indication of prior HAV infection.

Antibodies to capsid proteins are also elicited by inactivated or attenuated vaccines and both IgM and IgG responses are observed. The presence of these antibodies therefore cannot distinguish between an immune response due to vaccination and an immune response due to viral replication.

During replication, HAV synthesizes non-structural proteins from the P2 and P3 genome regions in addition to the capsid components. Antibodies against the P2 and P3 products could therefore potentially be used to differentiate immunity due to immunization from inactivated vaccines as opposed to immunity resulting from an infection involving active virus replication. Similar antigens have been shown to elicit an immune response within animals infected with a related picornavirus, foot-and-mouth disease virus (FMDV)[6]. Within countries in which FMDV is endemic, immunization of animals with inactivated vaccine is routine and therefore anti-capsid antibodies would not differentiate FMDV-infected animals. The presence or absence of antibodies against a viral protein from the P3 genome region, the RNA polymerase[A,7], is therefore used to determine whether an animal has FMDV due to vaccine failure or some other vesicular disease.

An approach to evaluate the host response to the non-structural antigens of HAV has been developed which utilizes immunoprecipitation of the protein products from *in vitro* transcription and translation of cloned cDNA derived from the non-structural genome regions[1]. These preliminary data indicated that patients with HAV

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infection have antibodies against the non-structural antigens (anti-P2 and anti-P3) in addition to antibodies against the capsid antigens. We have utilized this assay system to address the following questions. What is the natural course of anti-nonstructural antibody development during HAV infection? How does the presence of this antibody population correlate with the presence of anti-capsid antibodies within the general population? The answer to these questions will provide a basis for further development and potential use of this assay to differentiate HAV infection from immunization to HAV.

MATERIALS AND METHODS

Plasmids

The construction of the plasmids (pTHAV/P2 and pESHAV/P3) utilized for *in vitro* transcription-translation is similar to the plasmids which have been described previously[1]. They are composed of nucleotides 3208–4982 and 4977–7415 adjacent to the HAV or EMC (encephalo-myocarditis) 5' non-coding region and preceded by the T7 promotor.

In vitro transcription/translation and immunoprecipitation

The linearized DNA was transcribed to RNA with T7 RNA polymerase, phenol-chloroform extracted and ethanol precipitated. The resuspended RNA was then translated at 30°C for 90 min with rabbit reticulocyte lysate (Promega, Madison, WI, USA) in the presence of ^{35}S-methionine. Aliquots of the translation product (1.5 µl) were dispensed into 400 µl immunoprecipitation (IP) buffer (10mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100). Sera (2 µl) was added and the sample incubated overnight at 4°C. The next day, 25 µl protein-A/Sepharose beads (Pharmacia) were added and rotated at room temperature for 1 h. The protein A-bound antigen-antibody beads were then washed three times with IP buffer, followed by elution using IP buffer containing 4% sodium dodecyl sulphate. The eluted samples were then evaluated by scintillation counting. Preliminary evaluation of various sets of serum specimens using both the P2 and P3 non-structural antigens indicated that they yielded comparable immunoprecipitation results. For the purposes of this study, therefore, the P2 antigen was used to evaluate the presence or absence of anti-non-structural antibodies.

Determination of antibody response against the HAV capsid

The commercial HAVAB and HAVAB-M assays (Abbott Laboratories, North Chicago, IL, USA) were performed using the manufacturer's instructions to define prior or current infection with HAV.

Study populations

Serial serum samples were obtained from chimpanzees and cynomolgus monkeys after experimental oral or intravenous inoculation with HAV or after immunization with inactivated HAV. Human populations evaluated included: individuals who developed hepatitis A while participating in a hepatitis B vaccine efficacy trial; individuals involved in a common source outbreak of hepatitis A in an institution for the developmentally disabled (L. Polish, unpublished results); individuals aged 0–5 years and 25–35 years who participated in the 1976–1980 National Health and Nutrition Evaluation Survey, NHANES[3]; a similar aged cohort chosen from a seroprevalence study of Native Americans[4].

RESULTS

Previous data from an intravenously infected chimpanzee suggested that anti-non-structural antibodies developed rapidly and may be a sensitive marker for acute infection. However, the natural route of HAV infection is oral transmission. We therefore examined the development of anti-non-structural antibodies within animals who were exposed to HAV by the faecal–oral route compared to intravenous (i.v.) inoculation. A typical response to i.v. inoculation of HAV is shown in *Figure 1a*, with a marked rise in liver enzymes and a concomitant antibody response to the capsid and non-structural proteins as measured by HAVAB-positive status and immunoprecipitation of P2 antigen.

In contrast to this, *Figure 1b* illustrates the course of antibody development and liver enzyme elevations in a representative orally infected animal. As noted previously[5], orally infected animals tend to have a more restricted biochemical hepatitis compared to intravenously infected animals and the seroconversion to HAVAB-positive status is slightly delayed compared to the i.v. infected animals. The same general response also was found when these animals were evaluated for the appearance of P2 antibodies. The i.v. infected animals developed a rapid response to anti-P2 which paralleled the development of anti-capsid antibodies, while anti-P2

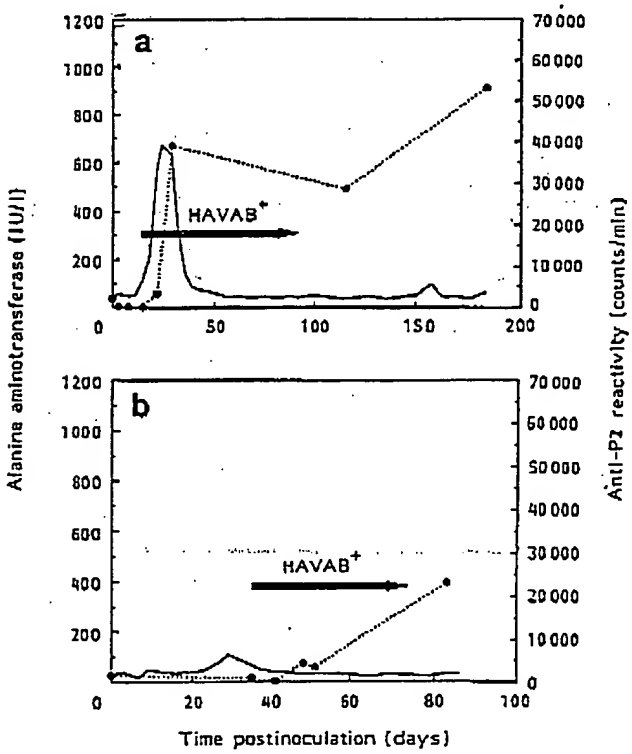

Figure 1 Serological response of chimpanzee to infection with HAV. a, Intravenous inoculation; b, oral inoculation. —, Alanine aminotransferase (ALT) values; ●, amount of radiolabeled P2 antigen immunoprecipitated

seroconversion within the orally infected animals developed gradually and increased over time.

We have evaluated nine experimentally infected chimpanzees who have developed signs of HAV infection, such as liver enzyme elevations and seroconversion to HAVAB-positive antibodies. All of these animals have also developed a positive response to the non-structural antigens as measured by P2 antibodies.

Serial serum samples were available from five homosexual men who acquired HAV infection while under observation in a hepatitis B vaccine efficacy study. Available serum samples were evaluated for the development of non-structural antibodies and correlated with other markers of hepatitis A. All of these individuals had high alanine aminotransferase (ALT) levels and clinical symptoms of hepatitis A, and all developed P2 antibodies (data not shown) that could be detected for up to one year after the peak of liver enzyme elevation. Within this group, HAVAB-positive status correlated well with the seroconversion to P2 positive antibodies.

Among patients involved in a common source outbreak of hepatitis A who were followed for 10 months after their infection, 75% had P2 antibodies during the acute illness and all had antibodies to non-structural proteins 10 months after their illness (*Table 1*).

Serial and paired serum specimens afford the opportunity to determine seroconversion to non-structural antibodies within individuals. For these studies, the cut-off value for the upper limit of normal was 2000 counts/min which was equivalent to twice the mean of known negative specimens. These data substantiate the conclusion that non-structural antibodies are a marker for active replication and infection with HAV and also suggest that the level of antibody as detected by immune precipitation increases after the acute phase of the illness.

To confirm the lack of P2 antibodies after vaccination with inactivated virus, two chimpanzees were immunized with 200 or 100 ng highly purified[8] and formalin-inactivated HAV (strain HAS-15). There was no detectable anti-P2 response after vaccination, although both animals seroconverted to HAVAB-positive status within 3 weeks. Wild-type virus challenge of the animal immunized with the 100 ng dose did not result in disease and no P2 antibodies were detected after challenge (data not shown).

Table 1 Anti-P2 reactivity of paired acute and convalescent phase antisera

Individual	Anti-P2 reactivity (counts/min)	
	Acute*	Convalescent
1	2 080	9 716
2	3 355	9 642
3	1 222	12 318
4	1 742	14 068
5	10 354	15 376
6	3 751	18 750
7	4 187	17 148
8	4 767	17 930
9	4 913	20 834
10	991	21 284
11	4 926	21 666
12	7 221	25 228

*Values below 2000 counts/min were considered to be negative for P2 antibodies based upon previous preliminary data[1]

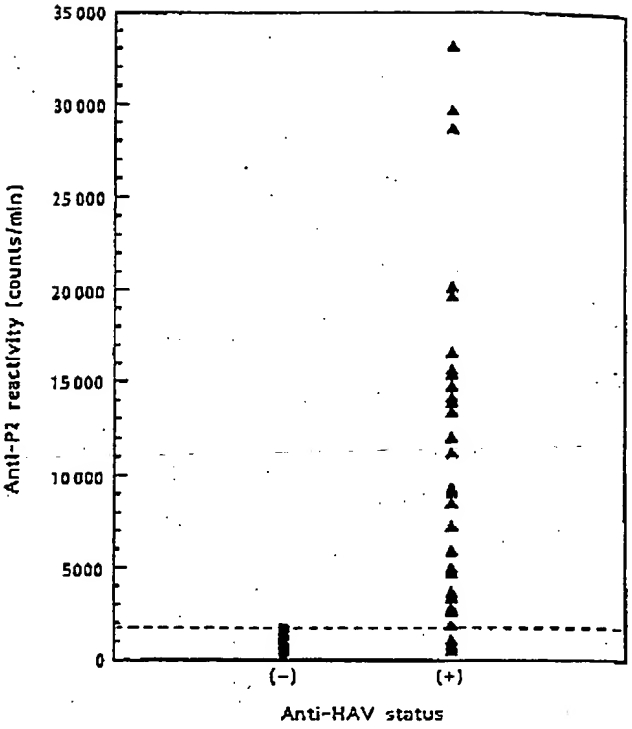

Figure 2 Anti-P2 reactivity of randomly chosen sera from across the USA. ●, Anti-P2 values obtained with HAVAB-negative samples; ▲, anti-P2 values obtained from HAVAB-positive samples

Specimens were chosen to evaluate the range of anti-P2 values within HAVAB-positive and negative individuals from two population-based studies (*Figures 2* and *3*). *Figure 2* illustrates the values obtained from a random sample of individuals from the USA[3]. Among HAVAB-negative individuals ($n = 49$) the average was 782 counts/min ± 273 in the anti-P2 assay. Two standard deviations above this level (1828 counts/min) was in general agreement with the cut-off used in the studies on patients with serial specimens. However, a wide range of values was found among the 42 HAVAB-positive individuals and 11 (26%) were negative for anti-P2.

Within the USA, limited numbers of individuals are exposed to HAV at an early stage. Stratification of the HAVAB-positive samples by age showed that, despite the limited number of samples within the 0–5 year old group, younger children (who generally experience asymptomatic infection) were more likely to have antibodies to capsid proteins in the absence of detectable P2 antibodies (*Figure 3a*). However, there also were a number of HAVAB-positive samples within the 25–35 year old group that had no detectable P2 antibodies. Native American populations tend to have high rates of HAV infection and HAVAB-positive specimens from 0–5 year old and 25–35 year old individuals were selected from a previous serosurvey of the Rosebud and Pine Ridge Sioux Indian populations[4]. In general, the level of P2 reactivity within HAVAB-positive children was lower (mean = 5417) than that found within the HAVAB-positive adult population (mean = 10497). However, there were still a significant number of individuals within each group who had anti-P2 reactivity which overlapped with the range of values found within HAVAB-negative individuals.

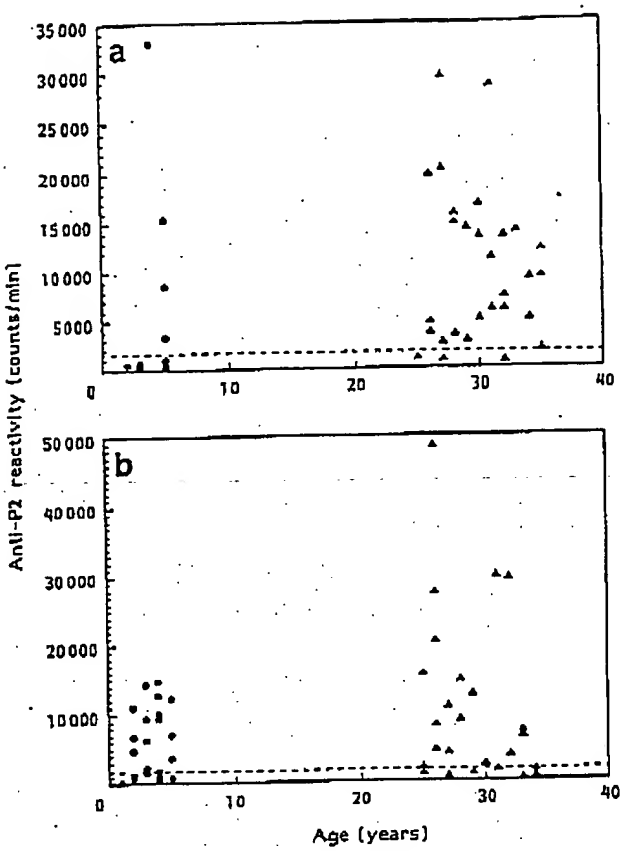

Figure 3 Scatter plot of anti-P2 reactivity of HAVAB-positive 0–5 year olds and 25–35 year olds. a, Randomly chosen sera from the USA; b, native Americans from the Pine Ridge and Rosebud Sioux Reservations in South Dakota

DISCUSSION

These investigations indicate that clinical illness following HAV infection results in the development of antibodies to both capsid and non-structural antigens. In cases where clinical or biochemical hepatitis A has been documented, non-structural antibodies were present in addition to capsid antibodies.

Despite the presence of non-structural antibodies following clinical disease, testing of HAVAB-positive and HAVAB-negative individuals from population-based studies showed that non-structural antibodies were not detectable in 26–39% of individuals who had serological evidence of a prior HAV infection. Our chimpanzee experiments suggest that, in infections where the ALT values are low or moderately elevated, the anti-P2 response is slower in development and of a lower final 'titre'. This may be reflected in asymptomatic infections, especially those that occur in children where the level of virus replication may be low. Thus, the anti-P2—negative results in persons with antibodies to HAV capsid proteins may reflect: exposure many years in the past; a lower initial response to active virus replication found primarily in asymptomatic infection; or test sensitivity.

Limited replication and asymptomatic infection would generate lower levels of both capsid and non-structural antibodies; subsequent exposure to HAV would then result in a boost of capsid antibodies without a simultaneous response to non-structural antigens due to the inhibition of virus replication. This differential response also may be related to the quantity of antigen produced during infection, which in turn affects the residual level of antibody to be detected. Recent data indicates that more capsid components are produced in larger abundance when compared to P2 and P3 products during cell culture replication[9] and this could affect the level and duration of the antibody response.

Alternatively, the sensitivity of a competitive inhibition assay such as HAVAB generally exceeds that of immune precipitation and the decreased sensitivity may account for the discrepancy. Preliminary analysis of antibodies against the P1 product as evaluated by the immunoprecipitation assay, indicates that individuals 'negative' for anti-P2 are also 'negative' for anti-P1, making lower sensitivity the most likely explanation for the differences.

The data indicate that antibodies against the non-structural antigens of HAV are generated after clinical or biochemical illness. This antibody population has the potential to be used to differentiate an antibody response due to immunization with inactivated vaccine from that resulting from illness, as it measures the host response to active virus replication. No antibodies to non-structural proteins should therefore be detected in an immunized person. The role of these antibodies in asymptomatic infections and the detection or duration of these antibodies within previously exposed individuals remains to be clarified.

ACKNOWLEDGEMENT

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EXHIBIT "C"

Journal of Medical Virology 40:76–82 (1993)

Antibody Response to Nonstructural Proteins of Hepatitis A Virus Following Infection

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The nonstructural proteins of hepatitis A virus (HAV), produced during active virus replication, are alternative antigens that could be used to differentiate disease from inactivated vaccine-induced antibodies. An assay based on immune precipitation of proteins translated from transcripts of the P2 region of viral cDNA was used to evaluate the development of antibodies after natural infection or vaccination. Antibodies against P2 proteins were found in all sera from clinical cases of hepatitis A following the acute phase. Chimpanzees vaccinated with inactivated or cell-adapted HAV had no detectable antibodies against P2 products, either before or after wild type virus challenge. A serosurvey of sera positive for total anti-HAV (HAVAB, Abbott Laboratories, North Chicago) suggested that some individuals had no detectable antibodies to the P2 antigen by immune precipitation. These results were attributed to the lower sensitivity of the immunoprecipitation assay, since antibodies to capsid proteins, as measured by immunoprecipitation, were also not detected in most of these sera.

KEY WORDS: HAV, immune precipitation assay, anti-P2, vaccine

INTRODUCTION

Hepatitis A virus (HAV), the causative agent of hepatitis A, is a member of the Picornaviradae family and until recently was classified as an enterovirus [Melnick, 1982; Gust et al., 1983]. Because of its unique genetic and physical characteristics, HAV has now been assigned to a distinct genus, hepatovirus, within the picornavirus family [Minor, 1991]. The basic architecture of HAV is a 27 nm icosahedral capsid that surrounds a 7.4 kb positive-stranded RNA genome. The coding part of the genome can be functionally delineated into three regions, P1, P2, and P3. The P1 region codes for the structural polypeptides of the capsid, whereas the P2 and P3 regions encode nonstructural polypeptides necessary for viral replication [Rueckert and Wimmer, 1984].

HAV infection is diagnosed by the detection of IgM or IgG antibodies to the capsid proteins [Bradley et al., 1977]. High levels of IgM antibodies to HAV (IgM anti-HAV) indicate acute infection whether it is asymptomatic or clinically apparent. IgM anti-HAV can generally be detected for 3–6 months after infection; however, IgG anti-HAV can be detected indefinitely and, in the absence of IgM anti-HAV, indicates prior HAV infection.

The development of inactivated vaccines to prevent hepatitis A infection [Flehmig et al., 1989; Andre et al., 1990; Ellerbeck et al., 1991] may lead to difficulties in diagnosis of acute HAV infection, especially asymptomatic infections. These vaccines elicit an IgG anti-HAV response, but after immunization a low-level IgM anti-HAV response may be detected. Currently, a person with inactivated vaccine-induced antibodies to HAV capsid proteins cannot be differentiated from a person with a prior HAV infection.

Serologic differentiation of immunity due to active virus replication from inactivated vaccine-associated immunity has been developed for foot-and-mouth-disease virus (FMDV), another picornavirus [Cowan and Graves, 1966]. The presence of antibodies against the RNA polymerase [Cowan and Graves, 1966; Robertson et al., 1983; Neitzart et al., 1991] has been used to determine whether a previously vaccinated animal had subsequently acquired FMDV infection. During replication, HAV synthesizes nonstructural proteins from the P2 and P3 genome regions, and antibodies against

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these proteins could be used to differentiate inactivated vaccine-induced immunity from an infection.

An immune precipitation assay that detects antibodies against nonstructural antigens of HAV has been developed utilizing products from in vitro transcription and translation of cloned cDNA [Jia et al., 1992]. Preliminary evaluation of selected sera suggested that nonstructural antibodies developed rapidly and might be a sensitive marker for acute infection. In this report, this assay was further characterized to determine its ability to identify both clinical and subclinical HAV infections and to define its usefulness in differentiating inactivated vaccine-induced immunity from natural infection.

MATERIALS AND METHODS

Plasmids

The two plasmids used for P2 or P3 transcription and translation (pTHAV/P2 and pE5HAV/P3) are the same as those described previously [Jia et al., 1992]. They are composed of nucleotides 3208–4982, and 4977–7415 adjacent to the HAV or EMC 5′ noncoding region and preceded by the T7 promotor within the pGEM vector (Promega, Madison, WI). Cleavage with *Nhe*I yielded a linear cDNA used for transcription and subsequent translation. The plasmid source for P1 transcription and translation was pT7-HAV4 [Harmon et al., 1991], which contains the entire HAV genome. Cleavage of this plasmid with *Sac*I produces a fragment that includes the T7 promoter, and the HAV 5′ noncoding region, and terminates at HAV nucleotide 2980, yielding a cDNA fragment encoding the P1 genome region.

In Vitro Transcription, Translation, and Immunoprecipitation

The linearized DNA was transcribed with T7 RNA polymerase, and nucleic acids were extracted with phenol-chloroform and precipitated with ethanol. Resuspended RNA was translated at 30°C for 90 min with rabbit reticulocyte lysate (Promega, Madison, WI) in the presence of [^{35}S]methionine. Aliquots of the translation product (1.5 μl) were dispensed into 400 μl of immunoprecipitation (IP) buffer (10 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100). Two microliters of serum were added and the sample was incubated overnight at 4°C. The next day, 25 μl of protein-A/sepharose (Pharmacia, Piscataway, NJ) was added, and the tubes were rotated at room temperature for 1 hr. The protein-A bound antigen-antibody beads were washed three times with IP buffer and were eluted using IP buffer containing 4% SDS. The eluted samples were evaluated by scintillation counting. Preliminary evaluation of various sets of serum specimens, using both the P2 and P3 nonstructural antigens, showed comparable IP results. Therefore, for this study, the P2 antigen was used to evaluate the presence or absence of anti-nonstructural antibodies. For these studies, the upper limit of normal was defined as 2,000 counts per minute (cpm). This value was equivalent to twice the mean of known negative specimens as determined in preliminary investigations [Jia et al., 1992].

Antibody Response to HAV Capsid Proteins

The commercial HAVAB and HAVAB-M assays (Abbott Laboratories, North Chicago, IL) were used according to the manufacturer's instructions to identify total anti-HAV and IgM anti-HAV, respectively.

Study Populations

Serial serum samples were obtained from chimpanzees experimentally infected with wild-type HAV by either oral or intravenous inoculation. In addition, four chimpanzees vaccinated (as described below) with either inactivated or cell-culture-adapted HAV, strain HAS-15 [Robertson et al., 1988], were evaluated.

Several populations with HAV infection were evaluated for antibodies to P2 antigens. Patients with documented HAV infection (symptomatic or asymptomatic) included (1) persons from whom serial blood specimens had been obtained as part of a hepatitis B vaccine efficacy trial [Francis et al., 1982] and who also developed hepatitis A; (2) persons involved in an outbreak of hepatitis A in an institution for the developmentally disabled (L. Polish, manuscript in preparation); and (3) patients with acute hepatitis A whose serum specimens were submitted for serologic testing. Other populations included persons aged 0–5 years and 25–35 years who were either positive or negative for total antibody to HAV. The samples were drawn from the National Health and Nutrition Examination Survey (NHANES II) conducted from 1976 to 1980 [National Center for Health Statistic, 1981], or from a seroprevalence survey conducted among American Indians who have a high endemic rate of HAV infection [Shaw et al., 1990].

Vaccination of Chimpanzees

Prototype vaccine was prepared from cell-culture-adapted HAV, strain HAS-15 (passage >60, FRhK4 cells). Virus was purified by isopycnic and rate zonal centrifugation as described previously [Robertson et al., 1988] and quantitated by its optical density at 260 nm [Rueckert and Pallansch, 1984]. The purified HAV was inactivated (HAS15-I) by incubating one part formalin with 4,000 parts of virus (1:4,000 formalin) at 37°C with stirring for 72 hr, followed by dialysis against phosphate-buffered saline (PBS). Inactivated HAV was adsorbed onto 1 mg of aluminum hydroxide. Cell-culture-adapted or attenuated (HAS-15A) virus used for vaccination was diluted in PBS and contained no adjuvant.

Four colony-reared and experimentally naive chimpanzees were vaccinated with inactivated HAS-15I (100 ng) or attenuated HAS-15A (100, 50, or 25 ng) HAV. The 100-ng vaccine doses were administered intramuscularly, while the 50-ng and 25-ng doses were administered intravenously. After vaccination, the chimpanzees were bled twice weekly to determine serum alanine aminotransferase (ALT) levels and anti-

bodies to HAV. Weekly liver biopsy specimens were obtained to monitor histopathologic changes and the presence of HAV antigen in liver cells. All animals were subsequently challenged intravenously with wild-type HAV (strain HLD-2) containing 10^6 marmoset infectious doses (K.A. McCaustland, unpublished results). Stool specimens were collected daily and prepared as 10% suspensions in PBS to determine HAV antigen by enzyme immunoassay (EIA) [Wheeler et al., 1986] and HAV RNA by polymerase chain reaction (PCR) amplification [Robertson et al., 1989].

RESULTS

Development of P2 Antibodies After Clinical Infection

Nine chimpanzees experimentally infected with wild-type HAV were evaluated for their antibody response to P2 antigens. All animals developed antibodies to capsid proteins (by HAVAB) and to the P2 antigens. The clinical, immunologic, and virologic patterns of HAV infection from seven of these chimpanzees (1149, 1181, 827, 1264, 1238, 1142, 825) have been included in a previous report [Margolis et al., 1988], while these characteristics for the other two chimpanzees (1394 and 1402) are shown in Figures 1A and 3D, respectively.

Since the natural route of HAV infection is through oral ingestion, we examined the development of P2 antibodies in those chimpanzees inoculated with HAV by the oral route compared with those inoculated by the intravenous route. A typical response to HAV infection after intravenous inoculation ($n = 6$ chimpanzees) included a marked rise in liver enzymes, preceded by antibodies against the capsid proteins, and an early antibody response to nonstructural proteins. A representative pattern of this response is shown in Figure 1A. Orally inoculated animals ($n = 3$ chimpanzees) tend to have a more restricted biochemical hepatitis compared with intravenously inoculated animals, and the antibody response to capsid antigens is slightly delayed compared with intravenously inoculated animals [Margolis et al., 1988] (Fig. 1B, also note change in scale on horizontal axis). Antibodies to P2 antigens after oral inoculation were detected after the resolution of the biochemical and histologic evidence of hepatitis (Fig. 1B).

Among four homosexual men who acquired HAV infection during the serologic follow-up phase of a hepatitis B vaccine efficacy study [Francis et al., 1982], all were symptomatic, although a wide range of liver enzyme elevations were observed (Fig. 2). Two cases had a biphasic liver enzyme pattern, and may represent relapsing disease [Gruer et al., 1982; Caredda et al., 1984; Raimondo et al., 1986; Sjogren et al., 1987] since there was no seroconversion to markers of hepatitis B virus infection, although markers of hepatitis C virus infection have not been tested. IgM anti-HAV and total anti-HAV were simultaneously detected in all patients, and all developed P2 antibodies (Fig. 2).

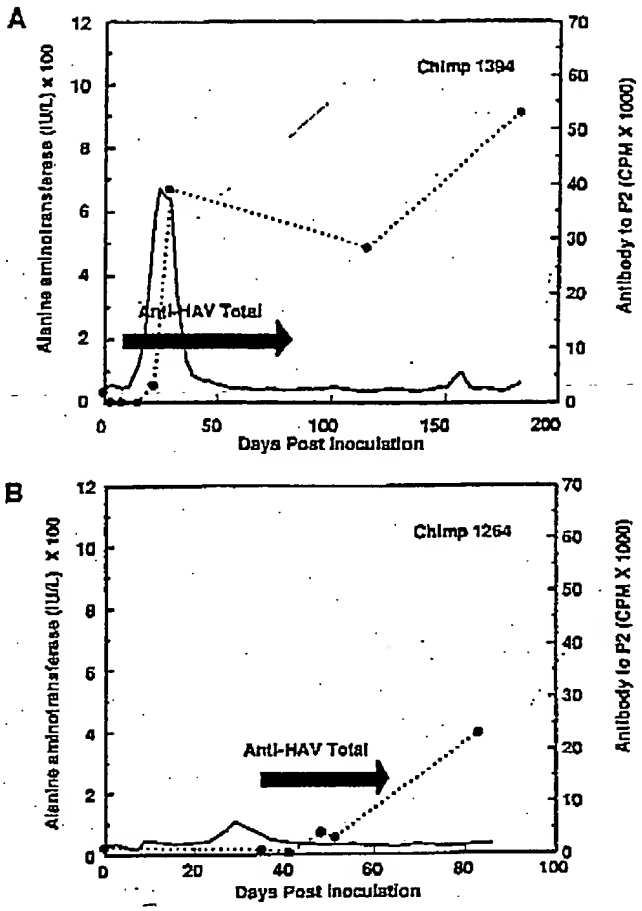

Fig. 1. Serologic and biochemical response of chimpanzees experimentally infected with HAV. (A) Intravenous inoculation with 1.0 ml of wild-type HAV, strain GA76 (22). (B) Oral inoculation via oral-gastric tube with 1.0 ml of wild-type HAV, strain AK76. The solid line indicates alanine aminotransferase values, while the closed circles and dotted line indicate the amount of radiolabeled P2 antigen immunoprecipitated. Immunoprecipitation of values above 2000 cpm were defined as positive for P2 antibody.

Detection of P2 Antibodies During Acute Illness

P2 antibodies in naturally infected humans and experimentally infected chimpanzees appeared to develop during or after liver enzyme elevations. Thirty-seven random serum specimens that were positive for IgM anti-HAV were evaluated for anti-P2, and 31 (84%) were positive. Antibody levels, as reflected by cpm of precipitated protein, varied widely. Forty-three percent (17) of the positive specimens had counts in the range of 2,000–10,000 and 45% (14) were greater than 10,000 cpm. A group of 12 people who were contacts of cases of hepatitis A during a common source outbreak and who were followed prospectively after receiving immune globulin were examined for the appearance of P2 antibodies. All became IgM anti-HAV positive, but only 75% had P2 antibodies at the time of IgM anti-HAV seroconversion. Ten months later, all of these individuals had developed high levels of antibodies to nonstructural proteins.

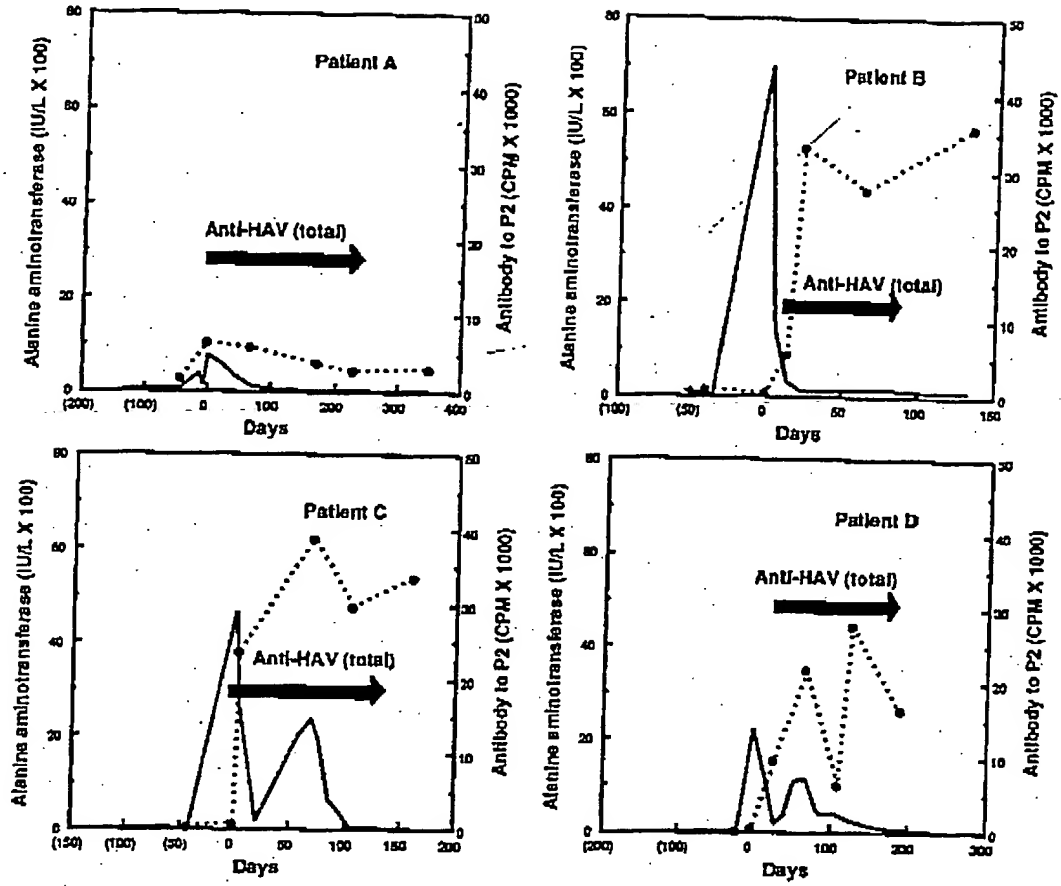

Fig. 2. Serologic events among four individuals with acute hepatitis A. Since the date of exposure was unknown in each case, day 0 refers to the date of the highest alanine aminotransferase (ALT) value. The solid line indicates ALT values, while the closed circles with dotted line indicates the amount of radiolabeled P2 antigen immunoprecipitated. The seroconversion to total anti-HAV (by HAVAB) positive status is illustrated by the solid arrow. Immunoprecipitation of values above 2,000 cpm were defined as positive for P2 antibody.

Antibody Response in Vaccinated Chimpanzees

Four chimpanzees were vaccinated with purified cell-culture-adapted HAV that was either inactivated with formalin and used with an alum adjuvant, or was considered to be attenuated and injected without adjuvant. The animals who received 100 ng of either the inactivated vaccine or the attenuated vaccine developed antibodies to the HAV capsid proteins between 14 and 21 days after vaccination (Fig. 3A and B). The animal receiving the 50 ng dose of attenuated HAV developed antibodies to capsid proteins 90 days after vaccination (Fig. 3C), and the animal receiving the 25 ng dose of the attenuated vaccine had no detectable antibodies to capsid proteins 218 days following inoculation (Fig. 3D). After challenge with wild-type virus, the three chimpanzees who had antibodies to capsid proteins were protected from infection (Fig. 3A–C) as shown by no elevation of liver enzymes, no histologic changes and no HAV antigen or RNA in stool specimens. A short duration IgM anti-HAV response was observed in the chimpanzee who was immunized with 100 ng of inactivated virus, and after challenge of the chimpanzee vaccinated with 50 ng of the attenuated virus; however, there was no evidence of viral replication by histopathology or virus excretion. The chimpanzee that was negative for antibodies to capsid proteins developed HAV infection (Fig. 3D).

No antibodies to nonstructural (P2) HAV proteins were detected in any of the animals who received inactivated or cell-culture-attenuated HAV. In addition, no animals who had antibodies to capsid antigens following immunization developed antibodies to nonstructural proteins after challenge with wild type virus. The chimpanzee that was anti-HAV negative rapidly developed antibodies against the nonstructural P2 antigen following infection with wild-type virus; these antibodies were detected at the same time as antibodies to capsid antigens.

Frequency of P2 Antibodies Within Selected Populations

Forty-nine randomly selected sera from individuals 1–5 years ($n = 21$) and 25–35 years ($n = 28$) who were negative for total anti-HAV (by HAVAB) were evaluated for anti-P2 by immune precipitation. The mean plus 2 standard deviations from these samples was equal to 1,828 cpm, a value similar to the 2,000 cpm cutoff obtained in previous studies [Jia et al., 1992].

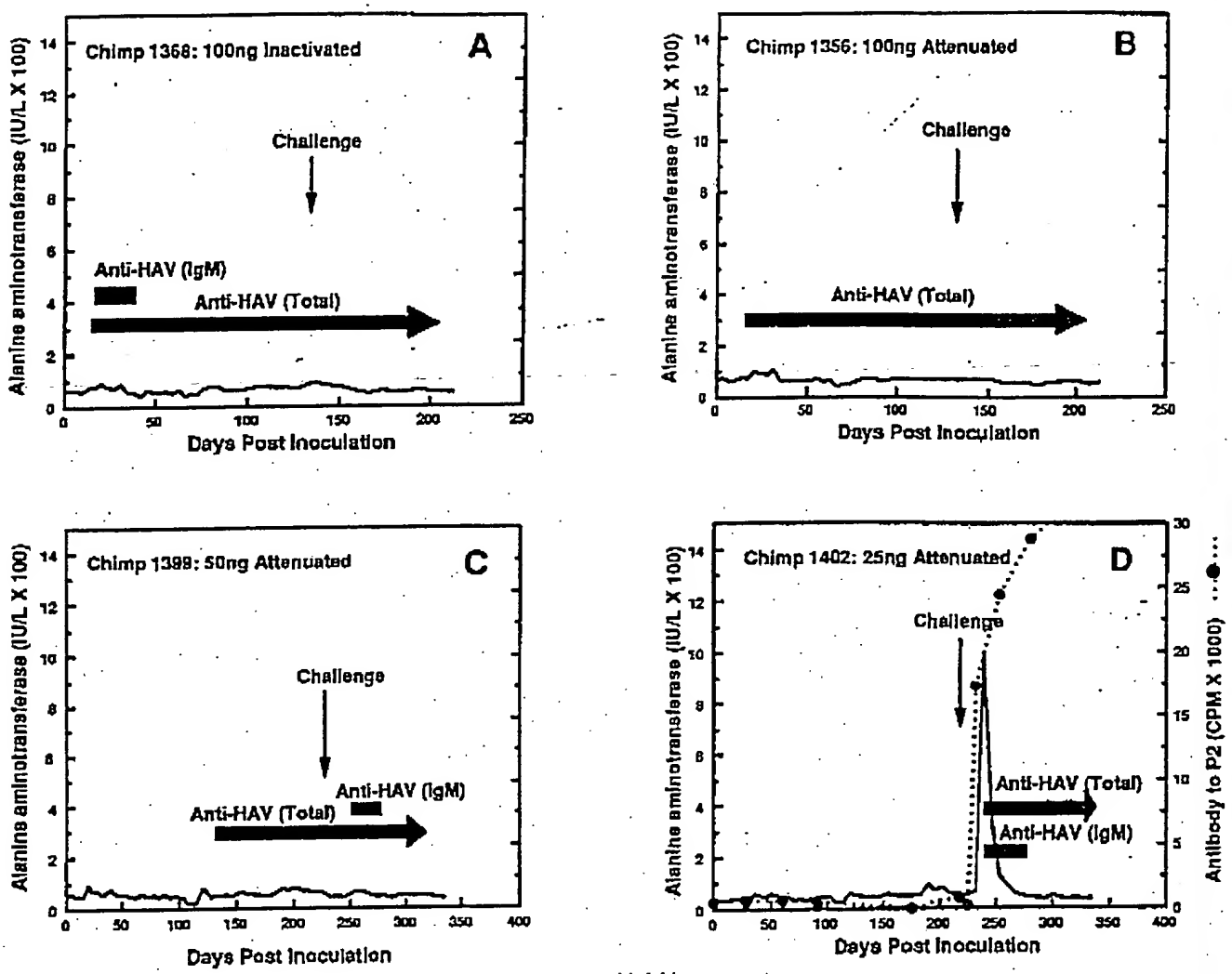

Fig. 3. Serologic analysis of chimpanzees immunized with 160 S purified inactivated or attenuated HAV (strain HAS-15). The solid line indicates alanine aminotransferase values (ALT), while the solid circles and dashed line indicate the amount of radiolabeled P2 antigen immunoprecipitated. The values for immunoprecipitation of P2 antigen in chimpanzees 1386, 1356, and 1399 all remained below 2,000 cpm (not shown). Seroconversion to total anti-HAV (by HAVAB) positive status is illustrated by the solid arrow, while detection of HAVAB-M antibodies is shown by the solid black bars. (A) Chimp 1368: Vaccinated with 100 ng of formalin inactivated HAV; (B) Chimp 1356: Vaccinated with 100 ng of attenuated HAV; (C) Chimp 1399: Vaccinated with 50 ng of attenuated HAV; (D) Chimp 1402: Vaccinated with 25 ng of attenuated HAV.

The distribution of antibodies to P2 antigens was examined in persons with serologic evidence of previous HAV infection. Within a comparable age group of 38 individuals randomly selected from a sample of the United States population who were positive for total anti-HAV (by HAVAB), 10 (26%) were negative for P2 antibodies. Similarly, among American Indian children 1–5 years ($n = 25$) and adults 25–35 years ($n = 25$) who were positive for total anti-HAV (by HAVAB) [Shaw et al., 1990], 19 (38%) were negative for P2 antibodies. All of these individuals represented prior infections as none of the sera were IgM anti-HAV positive. When anti-HAV positive individuals were stratified by age, persons in the 1–5 year age group were more likely not to have P2 antibodies (47%, 16/34) than persons in the 25–35 year age group (18%, 13/54); $X^2 = 4.00, P = 0.04$.

Evaluation of Assay Sensitivity

We attempted to determine whether the absence of detectable antibodies to P2 antigens in persons with antibodies to capsid proteins reflected test sensitivity or low antibody levels to the P2 antigens. Serum specimens from the American Indians that were positive for total anti-HAV capsid (by HAVAB) were tested for their ability to immunoprecipitate antigen derived from the P1 region (anti-P1). A strong correlation between P1 negative and P2 negative reactivity was found (Table I). Ninety percent (10/11) of the children who had negative P2 responses, also had negative P1

TABLE I. Detection of Anti-P1 and Anti-P2 by Immune Precipitation in Persons Positive for Total Anti-HAV†

	Children			Adults	
	P2 Ab(+)[a]	P2 Ab(−)[b]		P2 Ab(+)	P2 Ab(−)
P1 Ab(+)	11	1	P1 Ab(+)	16	2
P1 Ab(−)	3	10	P1 Ab(−)	1	6
		10/11 = 90%*			6/8 = 75%**

†Total anti-HAV (HAVAB, Abbott Laboratories, N. Chicago).
[a]Ab(+), antibody positive.
[b]Ab(−), antibody negative.
*$P = 0.0008$; Fisher's exact.
**$P = 0.001$; Fisher's exact.

responses, while 75% (6/8) of the adults who were P2 antibody negative were also P1 antibody negative.

DISCUSSION

Our data indicate that antibodies to nonstructural HAV proteins develop after clinical or biochemical hepatitis A infection. These antibodies were readily detectable by using a radioimmunoprecipitation assay and were present in addition to antibodies against the capsid proteins. The results support the concept that antibodies to nonstructural proteins may serve as markers for active replication and infection with HAV.

Animals immunized with inactivated or live-attenuated HAV had no evidence of nonstructural antibodies after immunization or challenge. These data clearly indicate that capsid antibodies are protective; among the immunized animals, the capsid antibody titer rose from 1:2 up to 1:200 or 1:400 after challenge, with no antibodies against the nonstructural antigens being detected. We assumed that the cell-culture-adapted HAV was attenuated, and that a low level of replication would occur. However, no viral excretion was detected by EIA or PCR, and no P2 antibodies were detected after inoculation of this live-attenuated virus. Two possibilities might account for these observations: (1) the cell-culture-adapted virus was "overattenuated" and no replication occurred, and the antibodies generated against the capsid proteins were due to immunization from the nonadjuvanted vaccine; (2) the level of virus replication was so low that an antibody response to the nonstructural antigens was not detected by using the current assay. Similar observations of decreased virus replication or undetectable virus have been reported for other candidate live-attenuated HAV vaccines obtained from prolonged cell culture passage [Provost et al., 1986; Karron et al., 1988].

Despite the development of P2 antibodies after clinical or biochemical hepatitis A infection, a substantial proportion of sera positive for total anti-HAV (by HAVAB) were negative for anti-P2. One explanation for this observation could be that antibodies against the P2 antigens are shorter lived than capsid antibodies. Within the American Indian population, where most HAV infections occur before 20 years of age [Shaw et al., 1990], the proportion of the population without detectable P2 antibodies did not increase with age. This observation indicates that anti-P2 is stable over several decades. On the other hand, the lack of P2 antibodies among children younger than 5 years of age suggests that asymptomatic infection may contribute to a poor antibody response to the nonstructural antigens. If the initial infection with HAV does not generate nonstructural antibodies, subsequent exposure to HAV would result in a boost of capsid antibodies with no virus replication. In addition, recent studies indicate that capsid proteins are produced in greater abundance than nonstructural virus proteins during replication of HAV in cell culture [Updike et al., 1991], and this may be relevant to virus replication and antibody responses in vivo.

We also determined that immune precipitation is probably not the most sensitive method for detecting antibodies to P2 antigens and may contribute to the inability to detect anti-P2 within individuals positive for total anti-HAV (by HAVAB). Among persons with total anti-HAV (by HAVAB), a competitive inhibition immunoassay, only 60% (30/50) had anti-P1 detected by immune precipitation. Among individuals whose P1 antibodies were not detected by immune precipitation, 80% (16/20) had no detectable P2 antibodies.

With further refinement of the antibody detection system, this antibody population could possibly be used to differentiate immunity due to inactivated vaccines from natural immunity. It also might be useful for identifying potential breakthrough infections occurring in persons who have been immunized with HAV capsid proteins. The role of these antibodies in asymptomatic infections and the detection or duration of these antibodies within the previously exposed individuals need to be evaluated further with more sensitive assays.

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The portion of this work performed at the University of Utah was supported by Grant AI26350 from the National Institute of Allergy and Infectious Diseases and by contract DAMD17-88-C-8122 from the US Army Medical Research and Development Command. We would like to thank G.M. McQuillan and C.N. Shapiro for identifying appropriate specimens from the NHANES II serum collection, L.B. Polish for followup serum, P.W. Coleman for helpful discussions regarding statistical analysis, and K. Krawczynski for histopa-

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EXHIBIT "D"

Host Antibody Response to Viral Structural and Nonstructural Proteins after Hepatitis A Virus Infection

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Subgenomic hepatitis A virus (HAV) RNA sequences were translated in vitro to produce proteins representing the structural (P1) and nonstructural (P2 and P3) domains of the viral polyprotein. These proteins were used as antigens to detect the presence of antibodies in sera from acute and convalescent humans and an experimentally infected chimpanzee. All infected individuals tested had antibodies that recognized uncleaved P1 proteins as well as nonstructural proteins. Antibodies in sera from infected individuals recognized conformation-dependent epitopes that were sensitive to SDS and heat treatment. Time-course studies of the experimentally infected chimpanzee showed that antibodies to the HAV proteins were detectable between 24 and 31 days after infection and persisted for >6 months. Human sera remained positive for antibodies to both structural and nonstructural antigens for at least 2½ years. The data suggest that HAV nonstructural proteins could be used as serologic markers for HAV diagnosis and for evaluating field trials of inactivated vaccines.

Hepatitis A virus (HAV) is a human pathogen that continues to pose a worldwide health problem, especially in developing countries. Local outbreaks can cause extensive disease, such as the 1987–1988 epidemic in Shanghai that resulted in >300,000 cases [1]. On the basis of its biochemical and biophysical properties, HAV is classified as a picornavirus [2]. It is distinguished from other members of this family, however, by having only limited sequence homology to members of any of the four classified genera and by the unique biologic characteristics of its growth in cell culture [3]. Whereas other picornaviruses undergo rapid, lytic replication cycles, HAV replicates slowly and asynchronously and establishes persistent infections.

Although HAV shares only minimal nucleotide or amino acid sequence homology with that of other picornaviruses, its overall genome organization is the same. A long 5′ noncoding region precedes a single open-reading frame that encodes one large polyprotein, which can be divided into P1, P2, and P3 domains. Computer-assisted sequence alignments predict further division of each domain into the characteristic picornavirus proteins from the P1, P2, and P3 regions [4]. The P1 region contains sequences for four viral capsid structural proteins. The P2 and P3 regions include numerous nonstructural proteins required for such activities as viral RNA replication and protein processing. HAV protein 3C has been demonstrated to have protease activity that can catalyze cleavage of at least some scissile bonds in the viral polyprotein [5, 6].

Since the successful adaptation of HAV to growth in cell culture [7] and since the construction of an infectious cDNA of HAV RNA [8], some progress has been made in understanding the molecular biology and growth properties of HAV [3]; detailed knowledge, however, and the molecular basis for its unique biologic properties remain obscure. Nevertheless, a commercial diagnostic kit for detecting HAV antibodies is available, and several preparations of both inactivated and attenuated vaccines are currently in field trial [9, 10]. It appears that even low levels of circulating humoral antibody are sufficient for protection against disease since administration of pooled human IgG provides solid albeit short-term protection. Very little is known, however, about the nature of the natural host immune response to infection [10]. Neutralizing antibodies appear just before or shortly after the onset of clinical symptoms and persist to provide apparently lifelong immunity. The roles of secretory immunity in protection are undefined as is the spectrum of viral proteins to which antibodies are directed.

Sera from animals convalescent from foot-and-mouth-disease virus (FMDV) infection contain high levels of antibody against a viral noncapsid protein that was identified as the viral RNA–dependent RNA polymerase [11, 12], derived from the COOH-terminal end of the P3 region [13]. It was reported that animals immunized with inactivated FMDV vaccine, in which the virus did not replicate, produced antibodies only to structural viral capsid components and not to the polymerase [14], although a subsequent report suggested that the polymerase was an internal and inherent component of viral particles and that sera from vaccinated animals did react with the polymerase antigen [15]. A more recent study clearly demonstrated that replication of FMDV in cattle,

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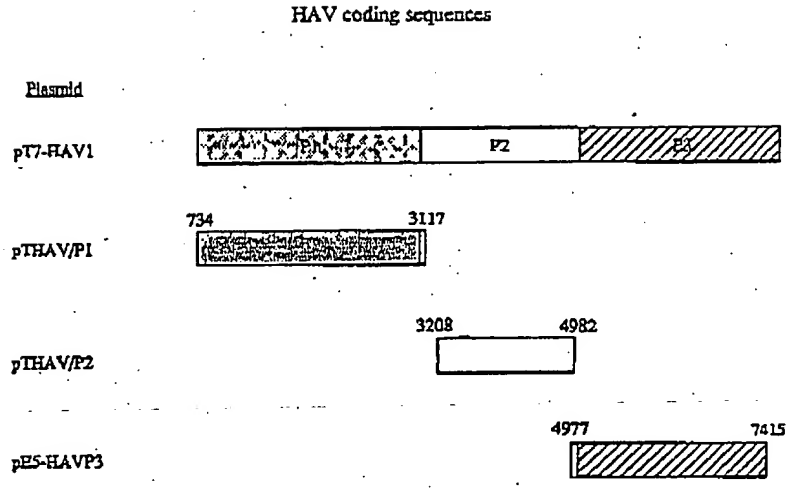

Figure 1. RNA transcripts of plasmids encoding hepatitis A virus structural and nonstructural protein sequences. Transcription by T7 RNA polymerase yielded transcripts that included hepatitis A virus nucleotides indicated by numbers. All but pE5-HAVP3 contain hepatitis A virus 5′ noncoding region; pE5-HAVP3 contains encephalomyocarditis virus 5′ noncoding region.

whether or not they exhibited symptoms of disease, elicited antibodies against at least four additional nonstructural proteins [16], and other investigators have recently shown that recombinant FMDV RNA polymerase, together with other nonstructural proteins, can be used in an enzyme-linked immunoelectrotransfer blot assay to detect antibodies to nonstructural proteins in the sera of persistently infected animals [17]. Sera from patients convalescent from other picornavirus infections, such as poliovirus or other enteroviruses, have not been examined for antibodies to nonstructural proteins. Here we report our studies of host antibody responses to both structural and nonstructural proteins after HAV infections in humans and in an experimentally infected chimpanzee.

Materials and Methods

Plasmids. The parental plasmid used for making all constructs was pT7-HAV1. Its construction has been described previously [18]. It contains the complete HAV cDNA sequences juxtaposed to a T7 promoter in pGEM-2 (Promega, Madison, WI). Transcription by T7 RNA polymerase generates plus-strand HAV RNA with five extra nucleotides at the 5′ terminus. The constructs prepared for this study are shown in figure 1. pTHAV/P1 and pTHAV/P2 contain T7 promoters adjacent to the HAV 5′ noncoding region (NCR) and extending through nucleotide 3117 for pTHAV/P1, or fused to nucleotide 3208 after the ATG and ending at nucleotide 4982 for pTHAV/P2. The latter construct was made by ligating the *XmnI-EcoRI* fragment (nucleotides 3208–4982), gel-purified from digested pT7-HAV1, to the parental plasmid cut with *XbaI* (nucleotide 749), filled in with Klenow fragment of DNA polymerase, and cut with *EcoRI*. pTHAV/P1 contains the entire P1 region and 5 amino acids from the NH_2-terminus of P2. pTHAV/P2 contains almost the entire P2 coding region; it is missing 32 amino acids from the NH_2-terminus and 6 amino acids from the COOH-terminus. pE5-HAVP3 has been described previously [19]. In this construct, the HAV 5′ NCR was replaced with the encephalomyocarditis virus (EMCV) 5′ NCR plus the first 10 amino acids of the EMCV leader sequence. This was fused to HAV sequences from nucleotide 4918 to the 3′ end. The resulting transcript codes for eight COOH-terminal amino acids of P2 and the entire P3 protein sequences of HAV.

In vitro transcription and translation. These procedures have been previously described [5]. Briefly, linearized DNA was transcribed by T7 RNA polymerase (Promega), and the nucleic acids were extracted with phenol, precipitated with ethanol, and finally resuspended in water. In vitro translation was done in rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]-methionine. Translation was done at 30°C for 90 min. The incorporation of [^{35}S]methionine was measured by trichloroacetic acid precipitation of a portion of each reaction, following the procedures recommended by the manufacturer.

Immunoprecipitation of translation products. Portions of the translation reaction were immunoprecipitated under denaturing conditions, as described previously [5]. Additional portions were immunoprecipitated under nondenaturing conditions. In the latter case, 2 μl of the translation reaction was diluted in 400 μl of immunoprecipitation buffer (10 m*M* TRIS-HCl, pH 7.4, 200 m*M* NaCl, 1 m*M* EDTA, 0.5% NP-40). Appropriate antisera were added (1.5 μl) and the sample was rotated overnight at 4°C. After the addition of 25 μl of 50% protein A–Sepharose (Pharmacia, Piscataway, NJ) suspension, the samples were rotated 1 h at 4°C. The protein A/antibody-antigen complex was collected by centrifugation, then washed three times with 0.5 ml of the same buffer. The complex was resuspended in 50 μl of SDS-PAGE sample buffer, boiled for 3 min, and clarified by centrifugation. Finally, 40 μl of the supernatant was analyzed by 10% SDS-PAGE and autoradiographed for 24–48 h or subjected to scintillation counting.

Serum samples. Eight HAV convalescent sera (including S8 and S9) were collected from Shanghai during the 1987–1988 outbreak. The sera were obtained 4–6 months after the onset of clinical disease. A University of Utah student from Shanghai became ill with HAV shortly after arriving in the United States in 1988. He donated blood samples at several time intervals during the subsequent 3 years. Seven HAV acute sera were obtained from local clinic patients in Utah. All of these patients

were positive for anti-HAV IgM, as determined by a commercial EIA for the detection of IgM antibody to HAV (HAVAB-M; Abbott, North Chicago) done by a local diagnostic laboratory. Three of our laboratory staff were identified as HAVAB-positive by routine screening. They had no history of hepatitis but were all from Asian countries where hepatitis A virus is endemic and likely had experienced subclinical childhood infections. Control sera were HAVAB-negative sera from American laboratory staff.

A series of chimpanzee sera were a gift from B. Robertson and H. Margolis (Hepatitis Branch, Center for Infectious Diseases, Centers for Disease Control, Atlanta). The chimpanzee was infected intravenously with the HLD2 strain of HAV. Liver alanine amino transferase (ALT) levels increased in the serum by day 17, peaked on day 28, and returned to normal by day 40. Antibody was detected by HAVAB assay on day 66 and remained positive for at least 1 year, when sampling stopped.

Rabbit sera raised against recombinant proteins containing HAV 2C and 3D sequences, produced in *Escherichia coli* and purified from SDS-polyacrylamide gels, have been described previously [5].

Results

Detection of antibodies to HAV proteins. To provide antigens for the detection and characterization of antibodies in serum samples, proteins composed of sequences encoded by each of the three regions of the HAV genome were synthesized in vitro in a rabbit reticulocyte lysate programmed with HAV RNAs transcribed from HAV cDNA constructs. Figure 1 shows diagrams of the transcripts used to generate proteins containing P1, P2, or P3 sequences. Almost all of the HAV protein sequences are represented in the translation products from these transcripts. Each transcript migrated in agarose gels as a single band with the mobility expected for its predicted molecular weight (not shown). Translation of the pTHAV/P1 transcript generated a single major protein of ~88 kDa (figure 2A, lane a). This transcript encoded HAV capsid protein sequences.

To test for the presence of antibodies against P1 protein sequences, we used two methods of immunoprecipitation. The first method involved complete denaturation of the antigen by boiling in SDS before addition of antiserum. This method had been demonstrated previously to efficiently recover proteins containing VP1 sequences by antiserum raised against VP1 immunogen prepared by elution from SDS-containing polyacrylamide gels [19]. We refer to this method as D-IP for immunoprecipitation under denaturing conditions. The second method was developed in an effort to retain conformation-specific epitopes that might be present in the protein, to which antibodies in the sera of infected animals might be directed. Consequently, samples were diluted with buffer containing only a mild, nonionic detergent (0.5% NP-40) at room temperature before addition of antiserum. We called this method N-IP.

Figure 2A shows the results of both methods of immunoprecipitation of the P1 capsid protein sequences by sera from a convalescent human HAV patient (S8) bled 4–6 months after the onset of clinical disease (lanes b and c) and from an experimentally infected chimpanzee bled 28 days after inoculation with HAV (lanes d and e). Both sera were positive for anti-HAV antibodies as determined by the commercial HAVAB assay. In both cases, efficient immunoprecipitation of HAV P1 protein occurred when the antigen was not denatured by SDS and heat (lanes c and e); no binding of antibodies present in the infected human or chimpanzee sera occurred when the antigen was first denatured with SDS and heat (lanes b and d). HAV P1 protein was not detected by control, uninfected human or chimpanzee sera under the same N-IP conditions (lanes f and g).

It was of special interest to determine whether acute or convalescent sera contained antibodies to HAV nonstructural proteins from the P2 or P3 regions. Antigens were prepared by in vitro translation of transcripts of pTHAV/P2 and pE5HAV/P3 (see figure 1), and the products were analyzed after D-IP and N-IP. Translation of the P2 protein sequences gave the pattern shown in figure 2B (lane a). A major band with the expected molecular mass of ~65 kDa was seen as well as a number of more rapidly migrating bands, which likely arise from aberrant internal initiation [19]. Immunoprecipitates from two human convalescent sera from the Shanghai epidemic (S8 and S9) are shown in figure 2B, both of which efficiently bound the P2 sequence antigen, only in the absence of SDS and heat denaturation (cf. lanes d and e with lanes b and c). Control sera from uninfected individuals precipitated no proteins by either IP method (not shown).

Similar results were obtained with P3 protein. The translation products from transcripts of pE5HAV/P3 have been described previously [19] and are shown in figure 2C (lane a). The largest protein represents the entire P3 region sequence, fused to a small portion (~2000 kDa) of the carboxy terminus of 2C. Proteolytic processing by the internal 3C sequences results in cleavage at the P2/P3 junction to generate the second major band, P3. Some secondary cleavages occur, as well as an extensive amount of aberrant internal initiation, to yield numerous additional polypeptides [19]. Almost all of these translation products were immunoprecipitated by human convalescent sera S8 and S9 (figure 2C, lanes d and e). Boiling the antigen in SDS markedly reduced its recognition by the antibodies present in the sera (figure 2C, lanes b and c), although a weak, specific immunoprecipitation still occurred, likely indicating a small fraction of antibodies directed against epitopes still present in the denatured protein.

Curiously, the complete translation product, 2C*P3 (the asterisk denotes an incomplete sequence), was not recognized by antibodies in these sera, whereas P3 and all smaller P3 sequence–containing polypeptides were efficiently precipitated. The reason for the interference by the partial 2C se-

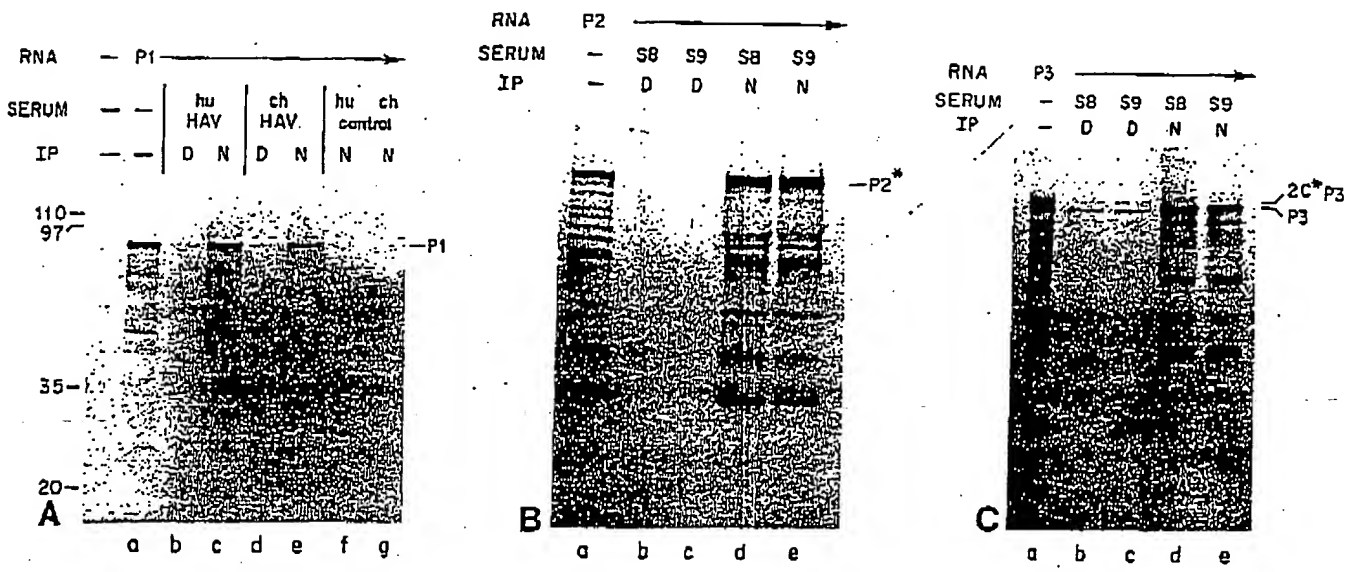

Figure 2. Immunoprecipitation (IP) of hepatitis A virus structural (P1, **A**) and nonstructural (P2 and P3, **B** and **C**) proteins, under denaturing (D) and nondenaturing (N) conditions, by human (hu) and chimpanzee (ch) sera, as indicated above each lane. Plasmids used: **A**, pTHAV/P1; **B**, PTHAV/P2; **C**, pE5-HAVP3. Lanes a, products analyzed by SDS-PAGE before immunoprecipitation. Human serum in A was S8; control sera were nonimmune human and preimmune chimpanzee sera.

quences is not known; possibly the terminal extension of 2C sequences affects the overall folding of the P3 protein, or possibly the protruding 2C sequences cover a major epitope. After denaturation, both 2C*P3 and P3 were immunoprecipitated at a low level (figure 2C, lanes b and c), although the short internal initiation products were not, and thus apparently lacked the reactive sequence.

The results shown in figure 2 demonstrated that sera from HAV-infected, convalescent patients contained antibodies to both structural and nonstructural HAV proteins. Exposure of both types of antigens to SDS, at high temperatures, however, nearly completely abolished their reactivity for these sera. To ensure that the SDS treatment did not generally inhibit the immunoprecipitation process, two rabbit sera that had been raised against SDS-denatured HAV proteins, 2C and 3D, were used to precipitate the P2 and P3 translation products, respectively. The anti-3D serum precipitated the native and denatured P3 protein nearly equally well (figure 3, lanes g and h), whereas the anti-2C serum bound denatured P2 protein significantly better than native (figure 3, lanes b and c). The two sera showed no cross-reactivity against P2 and P3 antigens (not shown), and preimmune sera from the same rabbits reacted with neither antigen (lanes d, e, i, j). Thus, sera raised against SDS-denatured proteins contained antibodies that bound SDS-denatured proteins, whereas sera from individuals infected with HAV contained antibodies that recognized only HAV proteins that have not been denatured with SDS and heat.

Kinetics of antibody response to various HAV antigens in an

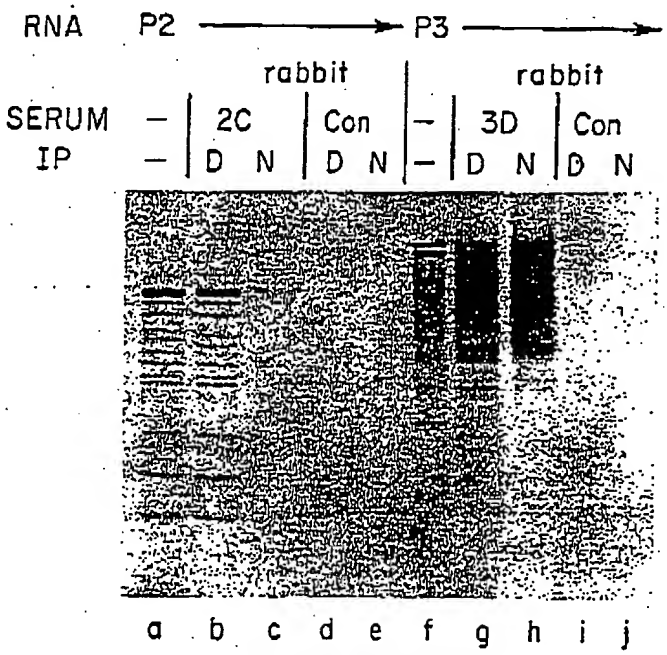

Figure 3. Immunoprecipitation (IP) of hepatitis A virus nonstructural proteins (P2 and P3), under denaturing (D) and nondenaturing (N) conditions, by control (Con) and immune rabbit sera. Sera were collected from rabbits immunized with recombinant proteins containing hepatitis A virus 2C or 3D sequences, which were purified from SDS-polyacrylamide gels. These and preimmune sera from the same rabbits were used to immunoprecipitate in vitro translation products of transcripts of pTHAV/P2 (lanes a–e) or pE5-HAVP3 (lanes f–j). Translation products before immunoprecipitation are shown in lanes a and f.

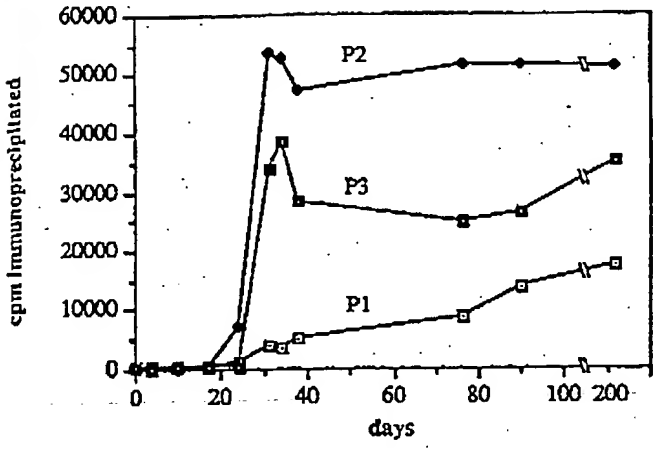

Figure 4. Appearance of antibodies to hepatitis A virus structural and nonstructural proteins in serum of experimentally infected chimpanzee. Serum samples obtained on indicated days after infection were used to immunoprecipitate, under nondenaturing conditions, [^{35}S]methionine-labeled in vitro translation products of transcripts from pTHAV/P1 (P1), pTHAV/P2 (P2), and pE5-HAVP3 (P3). Immunoprecipitates were collected and counted by scintillation spectroscopy.

experimentally infected chimpanzee. Serial bleeds from a chimpanzee experimentally infected with HAV were analyzed to determine the time course of appearance of antibodies to structural (P1) and nonstructural (P2 and P3) proteins. By commercial HAVAB test, anti-HAV antibody was first detected on day 66 after infection and remained present for at least 1 year. Serum liver enzyme (ALT) became elevated at day 17 after infection. The immunoprecipitation results for these sera are shown in figure 4. In this experiment, portions of the translation products immunoprecipitated by the N-IP method were assayed directly, before visualization by SDS-PAGE. Antibody against P2 protein was detected on day 24 and against P1 and P3 protein about 7 days later. The antibody titers to both P2 and P3 nonstructural proteins rose very steeply, reaching a maximum around 30–35 days and persisting until the last available bleed (day 203). Antibody titers to structural proteins, however, rose much more slowly and were still increasing over the time course of this experiment. Thus, antibodies to either the P2 or P3 nonstructural proteins may be a much more sensitive measure of infection at early times. Four sera from control, uninfected chimpanzees were negative for all three HAV antigens.

The autoradiographs of the SDS-PAGE analysis of the samples plotted in figure 4 are shown in figure 5. The pattern of immunoprecipitated proteins correlates well with and confirms the results deduced from direct assay of the samples.

It was of interest to compare relative antibody titers to the different HAV antigens produced by in vitro translation. To this end, fourfold serial dilutions of selected sera were prepared, and each was used to immunoprecipitate HAV structural (P1) and nonstructural (P2 and P3) protein sequences. Sera from an early (day 28 after infection) and a late (day 170) bleed of the chimpanzee and from an early convalescent (~10 weeks after onset of clinical symptoms) and a recovered (2½ years after infection) human patient were analyzed. Figure 6 shows examples of the dilution curves for the immunoprecipitations by 28-day chimpanzee serum and 10-week human serum. Since the curves indicate antigen excess (i.e., increasing antibody resulted in precipitation of more antigen), the amount of total antigen precipitated gives a measure of relative antibody titer for any given antigen. The data showed that antibodies to all three HAV protein

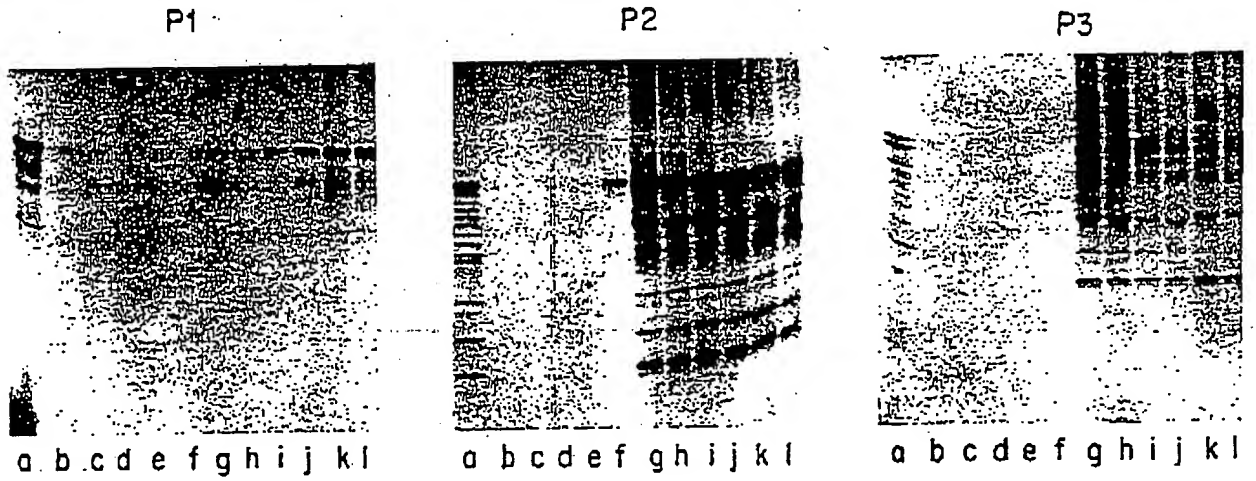

Figure 5. SDS-PAGE analysis of hepatitis A virus structural and nonstructural proteins immunoprecipitated under nondenaturing conditions by sera from an experimentally infected chimpanzee at different times after infection. Samples in lanes b–l are those plotted in figure 4. Lanes a, translation products obtained before immunoprecipitation.

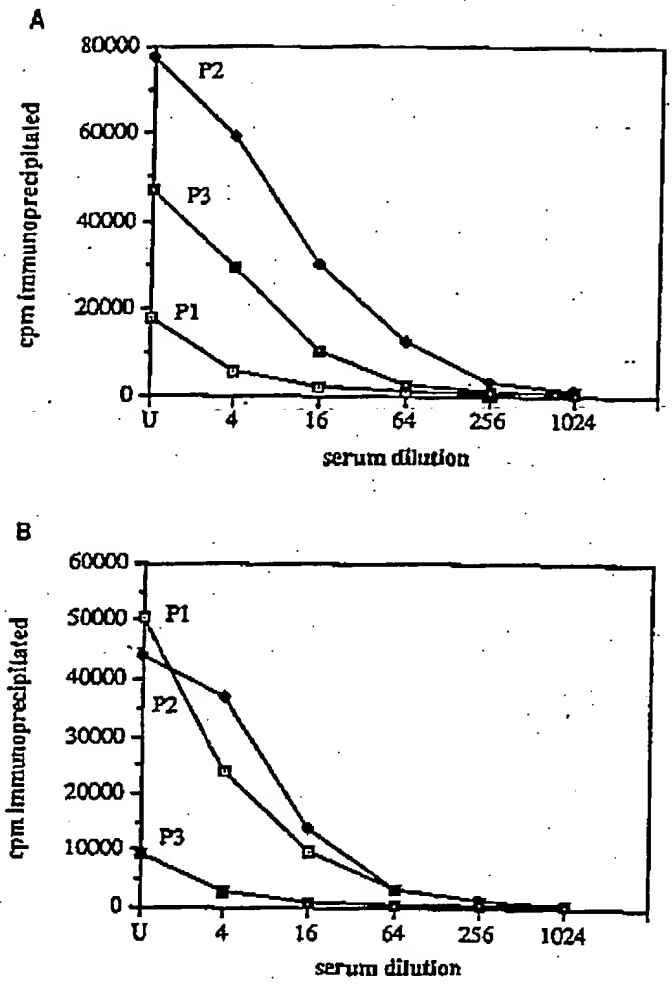

Figure 6. Immunoprecipitation of hepatitis A virus structural and nonstructural proteins by fourfold serial dilutions of serum: A, from chimpanzee 28 days after infection; B, from human 10 weeks after onset of clinical disease. U, undiluted.

sequences were present in all serum samples; the human samples had somewhat higher antibody titers to capsid (P1) proteins than the chimpanzee samples, whereas the chimpanzee sera had higher antibody levels against nonstructural (P2 and P3) proteins than the human. These trends may merely reflect individual variation in immune response, or they may be dependent on species differences in response to the infection or manner of infection or other factors. Since the specific activities of the three radiolabeled antigens made by in vitro translation were similar (although not identical), the amounts of each radiolabeled protein precipitated by a given serum are an approximate (but not precise) measure of the relative antibody titer to each antigen. Thus, for the chimpanzee, the antibody titers to P2 and P3 were higher than to P1; for the human samples, titers to P3 were somewhat lower. Table 1 shows the end-point dilutions of each serum sample required to give a positive immunoprecipitation signal. A signal was considered positive if it was two or more times the mean counts per minute of negative controls. One chimpanzee and one human HAV-negative sera were used as negative controls. Clearly, infected human or chimpanzee sera may also contain antibodies to protein structures not adapted by the in vitro translation products, and these would not be detected in this assay. Thus, the relative antibody titers measured here apply only to the antigens used in the analysis and not necessarily to the total antibody response to all HAV proteins.

Table 1. Antibody titers of human and chimpanzee sera against hepatitis A virus antigens.

Serum	P1	P2	P3
Chimpanzee			
Acute*	1:16	1:256	1:64
Convalescent†	1:256	1:1024	1:256
Human			
Early convalescent‡	1:64	1:64	1:4
Recovered§	1:256	1:16	1:4

NOTE. Titers are given as highest dilution that yielded positive immunoprecipitation (two or more times the mean counts per minute of negative controls).

* 28 days after infection.
† 203 days after infection.
‡ 10 weeks after onset of illness.
§ 2½ years after recovery.

Antibody response to HAV proteins in random clinical samples of HAV patients. Seven random sera that were HAV-positive by both standard HAVAB and HAVAB-M assays, obtained from a Salt Lake City diagnostic laboratory, and eight HAV convalescent sera collected 4–6 months after disease onset in Shanghai during the 1987–1988 outbreak were screened for antibodies to the different HAV proteins. Five control sera were obtained locally from individuals with no history of disease, these were antibody negative by commercial HAVAB test. The results are shown in figure 7. All of the 15 HAV sera had antibodies reactive with both P1 and P2 protein. Fourteen of the 15 HAV sera were antibody-positive for P3 proteins; the one P3-negative serum was from the Utah collection.

In addition, three sera from laboratory staff born in the Far East who were antibody-positive by HAVAB test but who had no history of disease were also analyzed. These individuals likely sustained subclinical infections >25 years ago. All three were positive for antibodies to P1; two had detectable antibody to P2 and P3, although titers were low (data not shown).

Discussion

Hepatitis A virus is unique among the Picornaviridae family in that its replicative cycle is prolonged, it does not inhibit

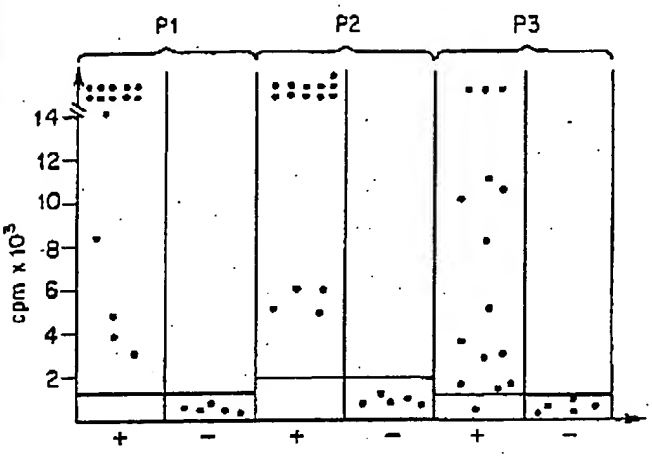

Figure 7. Immunoprecipitation of hepatitis A virus structural (P1) and nonstructural (P2 and P3) proteins by sera from 15 random clinical hepatitis A virus patients (+) and 5 controls (−). Immunoprecipitation was under nondenaturing conditions, and [^{35}S]methionine-labeled translation products represented the P1, P2, or P3 region as shown in figure 1. Immunoprecipitates were counted by scintillation spectroscopy. Each dot represents individual immunoprecipitation. Horizontal lines indicate lower limits of positive signal, defined as counts per minute two or more times the mean of the negative.

host cell macromolecular synthesis, replication-induced cytopathology is minimal to absent, and persistent infection is easy to establish in infected cells in culture [10]. Because of this noncytolytic, protracted replication, it has been difficult to identify and study the noncapsid proteins of HAV in infected cells in culture. Since the construction of an infectious cDNA of the HAV genome [8], it has been possible to transcribe cDNAs containing the P1, P2, and P3 regions of the genome in vitro and then to translate these transcripts in vitro. The HAV proteins so expressed were used as antigens to study the host antibody response after HAV infections in humans and a chimpanzee. Surprisingly, all sera examined from infected individuals contained antibodies against P1 capsid precursor proteins, as well as antibodies against nonstructural polypeptides from the P2 and P3 regions. These polypeptides included proteins representing the intact products of translation of the entire P2 and P3 transcripts plus aberrant internal initiation products and cleavage products. In each case, heat denaturation of the antigens in the presence of SDS before immunoprecipitation led to loss of antigen reactivity, suggesting that these antigens contain conformation-specific dependent or noncontiguous epitopes that are sensitive to denaturation. The facts that these polyclonal anti-P2 and anti-P3 antibodies, are present after natural HAV infections, are at levels equal to or greater than anti-P1 antibodies, and persist in humans for at least 2½ years have several important implications in studies of HAV pathogenesis.

First, the only currently available diagnostic test for HAV infection is the HAVAB competitive inhibition immunoassay, which measures antibody against intact virions. Because HAV grows poorly in cultured cells, the availability of this antigen is limited. The studies reported here show that antibodies against P2 and P3 HAV proteins can be detected early after infection, very shortly after serum ALT levels increased (figure 4). The levels of these antibodies remained high for as long as 6 months in the chimpanzee and for years in the human sera (figure 7). Since the P2 and P3 antigens can be easily synthesized in vitro, their use as diagnostic reagents is an attractive possibility for future HAV testing. Furthermore, the commercial diagnostic test cannot distinguish sera from persons vaccinated with inactivated HAV and sera from individuals with subclinical infections. As field trials of inactived HAV vaccine are begun, a diagnostic test based on the identification of antibodies to nonstructural proteins, which could arise only during a replicative infection, would be quite valuable to differentiate these individuals.

Second, the fact that anti-HAV antibodies other than antivirion antibodies have not been previously recognized in sera from HAV-infected individuals raises the question of what roles(s) these antibodies might play in immunity to HAV-induced disease. It is clear that passively transferred immune serum globulin provides protection from clinical disease for ≥3 months, and this passively transferred antibody produces only very low levels of neutralizing antibody in recipients [20]. The possible role(s) of anti-P2 and -P3 antibodies in protection should be considered in future studies of humoral immunity against HAV. Previous studies using monoclonal anti-HAV antibodies and viral escape mutants [21, 22] have shown that there are likely at least two important neutralization epitopes comprising VP1 and VP3 sequences in the HAV virion. Priming of neutralizing antibody responses has been observed in rabbits immunized with recombinant HAV VP3 and VP1 expressed in *E. coli* [23–25], and this priming perhaps represents T helper cell induction or memory. One must now consider the possible roles played by the antibodies directed against P1 proteins that are precursors to virions and by the anti-P2 and -P3 antibodies in both humoral and cellular immune mechanisms. This information will be critical to the design of future HAV vaccines.

The basis for the hepatocellular damage in HAV infection is not known, and it has yet to be established whether or not cytotoxic T cells play a role in immunity to HAV or in liver damage. Vallbracht et al. [26] have shown that human T cells from liver biopsies from HAV patients would lyse autologous HAV-infected skin fibroblasts. The antigens to which the T cells reacted were not identified. Since it is now clear that many antibodies in HAV-infected individuals are not antivirion, the roles of the entire spectrum of anti-HAV immune responses in the pathogenesis of HAV should be assessed.

Acknowledgments

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